The Functional Role of Palmitoylation of the 5-HT_{1A} Receptor

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Declaration

The thesis has been written independently and with no other sources and aids than quoted.

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ABBREVIATIONS:

AC - adenylate cyclase

APT – acyl protein thioesterase

AR - adrenergic receptor

cAMP - cyclic adenosine monophosphate

CHO – chinese hamster ovary

Ci – curie

CMV - cytomegalovirus

CNS - central nervous system

CRD - cysteine rich domain

Cys - cysteine

DMEM – Dulbecco's modified insect medium

DNA – desoxyribonucleic acid

8-OH DPAT – 8-hydroxy-(di-N-propylamino)-tetralin

DRM – detergent-resistant membrane subdomains

DTT – dithiothreitol

ECL - extracellular loop

EDTA - ethylenediaminetetraacetic acid

ER - endoplasmic reticulum

Erk - extracellular signal regulated kinase, the same as MAPK

5-HT - 5-hydroxytryptamine

FBS - fetal bovine serum

FCS – fetal calf serum

GABA - gamma-amino butyric acid

GDP - guanosine diphosphate

GFP – green yellow fluorescent protein

GPCR - G-protein coupled receptor

GPI - glycophosphatidilinositol

G–protein - heterotrimeric GTP binding protein

GTP - guanosine triphosphate

GTPγS - guanosine triphosphate with the γ-phosphate replaced by the sulphate

ICL - intracellular loop

INCL – infantile neuronal ceroid lipofascinosis

IP – inositoltriphosphate

IPTG - Isopropyl-b-D-thiogalactopyranoside

LH/hCG – luteinising hormone/human chorion gonadotropin

MAPK - mitogen-activated protein kinase, the same as Erk

OP - Optiprep™

PAGE - polyacrilamide gel electrophoresis

Pal-CoA - palmitoyl coenzyme A

PAT - palmitoylacyltransferase

PCR - polymerase chain reaction

pfu - plaque forming units

PIP – phosphatidilinositol triphosphate

PLC - phospholipase C

PM - plasma membrane

pPolh – polyhedrin promoter

PPT - protein palmitoyl thioesterase

PS – penicillin-streptomycin

PSD - postsynaptic density

RGS –regulator of G-protein signaling

RNA – ribonucleic acid

RT – room temperature

SDS - sodium dodecyl sulphate

Ser - serine

Sf.9 – *Spodoptera frugiperda* insect cells

SRE - serum response element

TEMED – tetraeminethylendiamine

TfR – transferrin receptor

TM - transmembrane

VIP - vasoactive intestinal peptide

WT – wild type

X-gal - 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

YFP – yellow fluorescent protein

1. INTRODUCTION

Adequate reaction of any living organism to environmental changes is essential for its survival. To this end, a variety of different control and regulatory systems has been developed during the evolution. In the multicellular organisms this task is sophisticated, as it is necessary to provide a concerted and coordinated action of the cells within the body. It is not surprising that aberrations in intercellular signal transduction pathways often lead to the severe disorders. Accordingly, proteins participating in signal transduction represent the most common targets for the medications.

To interpret extracellular signals, cells maintain a diversity of surface receptors that respond specifically to individual stimuli. The five major classes of the receptors include (1) cytokine receptors responsible for the crosstalk between cells, (2) integrin receptors that mediate interaction of the cells with the extracellular matrix, (3) receptor tyrosine kinases that serve as receptors for the growth factors and thus controlling cell proliferation and maturation, (4) ligand-gated ion channels that transmit various signals via modification of the permeability of the plasma membrane for certain ions and (5) G-protein coupled receptors (GPCR). The later class of receptors mediates a large variety of physiological responses via interaction of the receptor with the heterotrimeric G-proteins followed by activation of various second messengers.

1.1 G-Protein Coupled Receptors

The G-protein-coupled receptors (GPCRs) represent the superfamily of proteins that are able to recruit and regulate the activity of the intracellular heterotrimeric GTP binding proteins (G-proteins). GPCRs govern the reactions to a wide range of signals, including

odours (Gaillard et al., 2004), taste (Hoon et al., 1999; Zhang et al., 2003), light (Filipek et al., 2003), hormones and neurotransmitters. GPCRs represent one of the largest protein families in the human genome (Lander et al., 2001), and the largest family of the membrane receptors. These receptors are involved in regulation of various biological functions and represent one of the most important targets for therapeutic treatment.

All GPCRs possess seven transmembrane domains linked by the alternating intracellular (ICL1-ICL3) and extracellular (ECL1-ECL3) loops, with the amino terminus located on the extracellular side and the carboxyl terminus on the intracellular side (Fig 1.1). The extracellular N-terminus and loops, together with transmembrane domains, are known to be involved in the interaction of the receptor with the ligand (Gether, 2000). Intracellular parts of the GPCR, including the C-terminal domain and the intracellular loops (in particular the ICL2 and the ICL3), are known to be important for the G-protein recognition and activation (Kobilka, 1992; Savarese and Fraser, 1992; Wess, 1997).

Although the GPCRs do not show the overall sequence homology, a significant homology is found within the defined subfamilies (Gether, 2000). The three major subfamilies include the receptors related to the rhodopsin and the β_2-adrenergic receptor (family A), the receptors related to the glucagon receptor (family B) and the receptors related to the metabotrophic neurotransmitter receptors (family C). The receptors for yeast pheromones form two minor unrelated subfamilies (family D and E), and four cAMP receptors from *Dictiostellum discoideum* constitute another unique GPCR subfamily (family F) (Gether, 2000; Kolakowski, 1994).

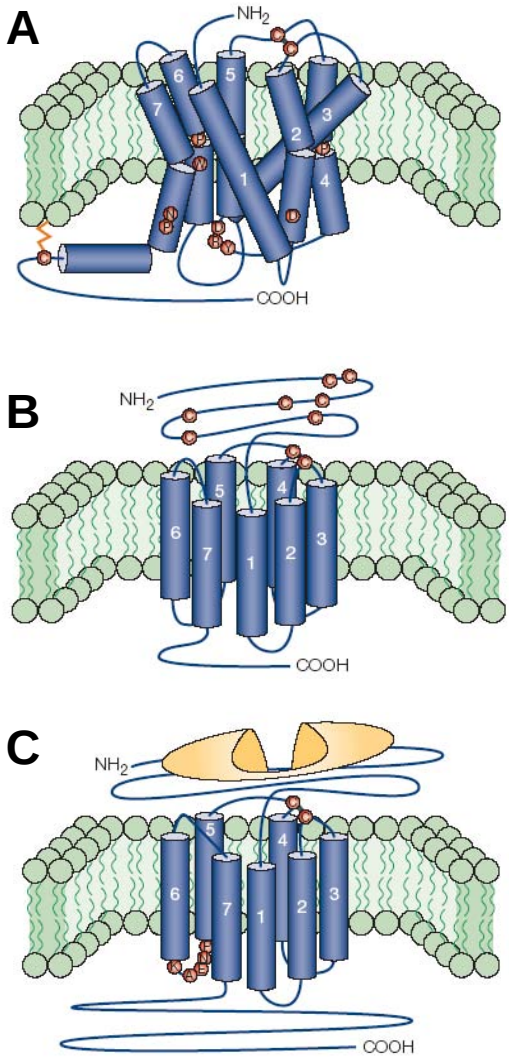


Figure 1.1. The Three Major Families of the GPCR. The **Family A** (upper panel) receptors are characterized by several highly conserved amino acids (red circles) and a disulphide bridge that connects the first and second extracellular loops (ECL). An orange zigzag represents modification with palmitic acid that is present in most Family A GPCR. Determination of the crystal structure indicated that the transmembrane domains (TM) of family 1 receptors are 'tilted' and 'kinked' as shown, due to the presence of amino acids such as proline that distort the helical transmembrane domain. **Family B** GPCRs (middle panel) are characterized by a relatively long amino terminus, which contains several cysteines that form a network of disulphide bridges. **Family C** (lower panel) receptors are characterized by a long amino terminus and carboxyl tail. A unique characteristic of these receptors is that the third intracellular loop is short and highly conserved (Nature Reviews Drug Discovery GPCR Questionnaire Participants, 2004)

The **Family A** (rhodopsin/β_2 adrenergic-like receptors) represents the largest GPCR family. It includes the receptors for biogenic amines (adrenergic, serotonin, dopamine, muscarinic, histamine), opsins, some peptide ligands (endothelin, neurotensin, oxytocin, vasopressin etc), adenosine, opioid and many other compounds. The olfactory receptors also belong to this family (Gether, 2000). The common structural feature of the family A receptors is the relatively short (about 40 aa) extracellular amino terminus (Fig. 1.1). In

addition, a majority of the receptors from this family has palmitoylated cysteine(s) in the carboxyl-terminal tail (Qanbar and Bouvier, 2003) causing formation of a putative fourth intracellular loop (Palczewski et al., 2000). The transmembrane domains of the family A receptors are critically involved in the ligand binding (Gether, 2000). The binding crevice for the small ligands is formed exclusively by the amino acid residues of the transmembrane domains and is deeply buried in the receptor molecule (Tota and Strader, 1990). In contrast, mutational mapping of the ligand-binding sites in many of the peptide family A receptors has demonstrated critical involvement of the N-terminus and the extracellular loops for binding of the larger peptide ligands along with the crevice formed by the transmembrane domains (Gether, 2000).

The **Family B** receptors include approximately 20 different receptors for neuropeptides, for example the vasoactive intestinal peptide (VIP), the calcitonin, the glucagon and others. The common feature of the family B receptors is a large (about 100 aa) extracellular N-terminus, containing several cysteines, presumably forming a number of disulfide bridges (Fig. 1.1). Similar to the peptide receptors belonging to the family A, the binding sites for the ligands in the family B involve the extracellular domains. The large amino terminus of the family B receptors seems to play a prominent role in the ligand binding, although the additional interactions of the ligand with the extracellular loops are also sufficient (Buggy et al., 1995; Stroop et al., 1996).

The **Family C** receptors include the metabotrophic glutamate and $GABA_B$ receptors, the calcium receptors, the pheromone and the taste receptors. These receptors possess a very long (about 600 aa) amino terminus and a short and highly conserved third intracellular loop (Fig. 1.1). The large amino terminus forms a ligand-binding site (Conn and Pin, 1997; O'Hara et al., 1993).

An increasing number of studies have demonstrated that the GPCRs do not act as monomeric proteins, but often form functional homo- and/or heterooligomers (Angers, 2002; Ayoub et al., 2002; Germain-Desprez et al., 2003; Issafras et al., 2002). The existence of such oligomers has been confirmed by different experimental approaches including those in the living cells (Angers et al., 2001). The oligomerisation has been suggested to play a role in the various aspects of receptor biogenesis and function. Several studies demonstrated that the GPCR dimerisation can occur in the endoplasmic reticulum and thus can be important for the correct receptor traffic (Karpa et al., 2000; Margeta-Mitrovic et al., 2000; Zhu and Wess, 1998). For some GPCRs, ligand-modulated dimerisation at the plasma membrane has been proposed (Angers et al., 2000; Cvejic and Devi, 1997). Interestingly, heterooligomerization between the distinct receptor subtypes has been shown to result in pharmacological properties different from those of the individual receptors (Gomes et al., 2000; Jordan and Devi, 1999; Maggio et al., 1999; Yoshioka et al., 2001). Moreover, heterooligomerization can change a downregulation mode of GPCRs (AbdAlla et al., 2000; George et al., 2000; Jordan and Devi, 1999) and the G-protein coupling (George et al., 2000). Thus the discovery of the GPCR oligomerisation could have a considerable impact for the drug development.

1.2 G-protein Mediated Signaling

According to the generally accepted model, the G-protein coupled receptors exist in the equilibrium between the inactive and the active states (Riitano et al., 1997; Scheer et al., 1996; Schwarts, 1995). In the active states, the receptors can activate the heterotrimeric G-proteins, while in the inactive states they are silent in terms of signaling.

In the absence of the external stimuli, the equilibrium of the receptor states is shifted towards the inactive conformations (Leurs et al., 1998), however a certain fraction of the receptors spontaneously assumes the active states. Ligand binding to the receptor can shift the equilibrium towards the active (agonist) or the inactive (inverse agonist) state.

The heterotrimeric G-proteins are composed of the α-subunit (Gα) and the dimer of the βγ-subunits (Gβγ) (Fig 1.2). Activation of the receptor results in the replacement of the GDP bound to the Gα subunit by the GTP molecule. After that, the heterotrimer can dissociate into the separate Gα and Gβγ subunits (Hamm, 1998; Hepler and Gilman, 1992; Lambright et al., 1996; Mixon et al., 1995; Wall et al., 1995), although some recent data suggest that the G-protein activation may also involve the subunit rearrangement rather then the dissociation (Bunemann et al., 2003).

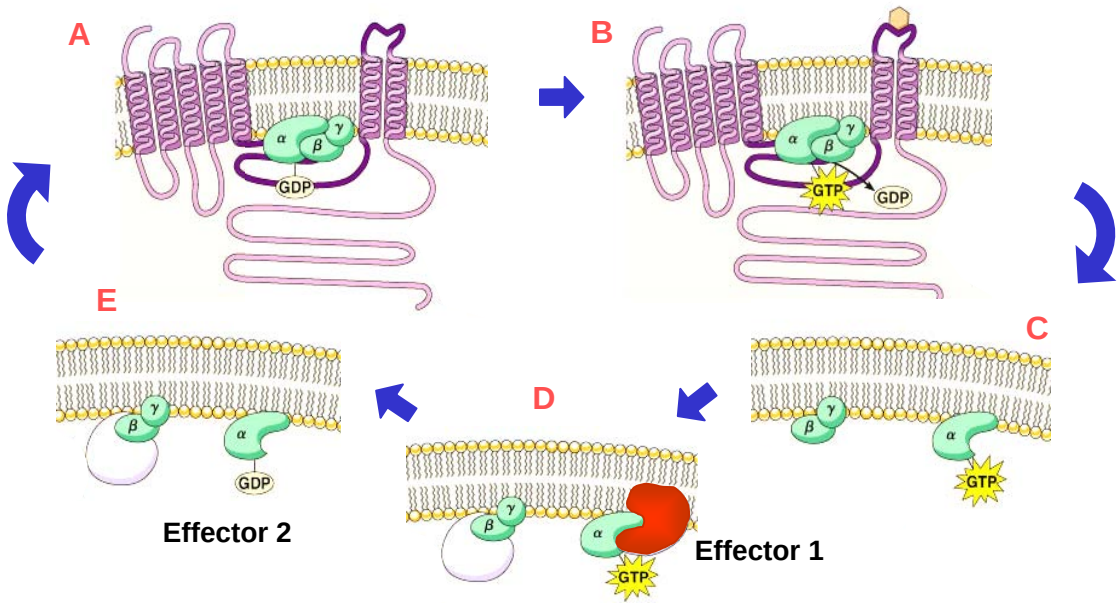
The activated Gα and Gβγ subunits can regulate the activity of the downstream effectors such as adenylate cyclases, phospholypases, ion channels and etc. The termination of the signal is achieved by hydrolysis of the Gα-bound GTP due to the intrinsic GTPase activity as well as by interaction with specific regulatory proteins (i.e. RGS proteins) (Doupnik et al., 1997; Druey et al., 1996; Watson et al., 1996). This leads to the inactivation of the Gα and to the formation of heterotrimer that is readily available for another round of the activation (Fig. 1.2).

To date, all known G-proteins are divided into four main classes according to the amino acid sequence similarity of their Gα subunits.

The **G_s protein** class includes $G\alpha_s$ and olfactory $G\alpha_{olf}$. The common property of $G\alpha_s$ is stimulation of the adenylate cyclases (AC) leading to the increase of cAMP concentration. In addition, the $G\alpha_s$ proteins can stimulate L-type calcium channels

(Mattera et al., 1989) and inhibit cardiac voltage-dependent sodium channels (Schubert et al., 1989). The G_{olf} protein is expressed exclusively in the olfactory neuroepithelium and serves to link odorant receptors with the olfactory-specific form of the adenylate cyclase.

Figure1.2. The G-protein activation cycle. A. Inactive GDP-bound Gα subunit assembles with the Gβγ complex. **B**. Activation of the GPCR by the external stimulus promotes exchange of the GDP to the GTP and thus activation of the G-protein heterotrimer. **C.** Activated G-protein heterotrimer dissociates from the receptor. At the same time, the GTP-bound Gα subunit dissociates from the Gβγ complex. **D**. The Gα subunit and the Gβγ complex activate the respective effectors. **E.** The Gα subunit hydrolyses the GTP to the GDP, becomes inactive and builds the complex with the Gβγ subunits.



The class of **G_q proteins** includes the ubiquitously expressed $G\alpha_q$ and $G\alpha_{11}$ proteins, the $G\alpha_{14}$ (present in the lung, kidney and liver tissues), and the $G\alpha_{15}$ and $G\alpha_{16}$

subunits expressed in the myeloid cells and the lymphocyte cells (Simon et al., 1991). The G-proteins of this class activate the phospholipase C, an enzyme that catalyses hydrolysis of the phosphatydinositol 4,5-biphospate on the two second messengers, the diacylglycerol (DAG) and the inositol 1,4,5-triphosphate (I1,4,5-P). DAG activates the protein kinase C, while I1,4,5,P promotes release of the intracellular calcium and thus activation of the phospholipase A2. Also $G\alpha_q$ directly stimulates tyrosine kinase activity in the lymphoma cells (Bence et al., 1997). In addition, the $G\alpha_{q/11}$ proteins are shown to inhibit neuronal inwardly-rectifying potassium channels (Firth and Jones, 2001).

The class of **$G_{i/0}$-proteins** includes the nearly ubiquitously expressed $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ and $G\alpha_0$ proteins as well as the brain- and the adrenal platelets-specific $G\alpha_{z,}$. It also includes the $G\alpha_t$ and the $G\alpha_g$ expressed in the retina and the taste buds, respectively. The common property of the $G_{i/0}$-protein family is the inhibition of the adenylate cyclase activity. Moreover, the G_i and the G_0 proteins have been shown to activate the G-protein coupled inwardly-rectifying potassium channels (GIRK) (Yatani et al., 1988), to inactivate the L, N and P/Q types of calcium channels (Dolphin, 2003), to stimulate the phospholipase C and the phospholipase A2 and to activate the mitogen-activated protein kinase Erk1/2 (Cano and Mahadevan, 1995). The $G\alpha_t$ subunits stimulate the cGMP-specific phosphodiesterase in the retinal rods and cones (Stryer, 1986).

The **G_{12} protein** class is composed by the G_{12} and the G_{13} proteins, that show the relatively low sequence homology with the other G-proteins. These proteins are involved in the modulation of small GTPases activity and thus in regulation of the cell morphology (Buhl et al., 1995; Kozasa et al., 1998; Suzuki et al., 2003). The $G_{12/13}$ proteins are also

shown to activate an extracellular signal regulated activated kinase (Voyno-Yasenetskaya et al., 1996) and the Na^+/H^+ exchange (Voyno-Yasenetskaya et al., 1994).

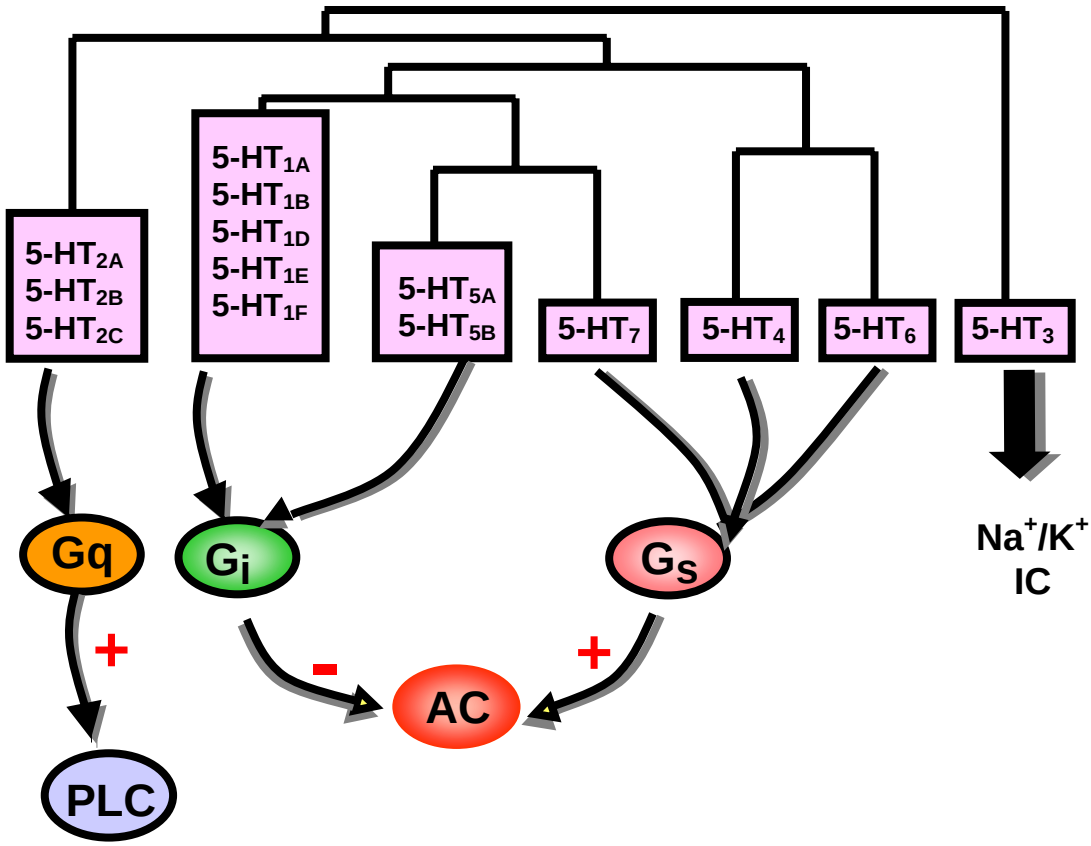
The **β and γ subunits** of the G-proteins can be considered as functional monomers due to their continuous tight interaction. At least five isoforms of β subunits (Watson et al., 1994) and six of γ subunits (Cali et al., 1992) have been discovered to date. These isoforms can form different combinations. The βγ-complex acts not only as an anchor for Gα subunits to form the functional heterotrimer, but also directly mediates a number of specific responses, such as the regulation of the GIRK channels, the activation of the phospholipases C and A_2, the activation of the MAPK and the modulation of some isoforms of adenylate cyclase.

1.3 Serotonin (5-hydroxytryptamine or 5-HT) Receptors

The neuromodulator serotonin is involved in regulation of the variety of sensory and motor functions within the CNS and in the periphery. The serotonergic innervation originates from the caudal raphe nuclei in ventral part of the brainstem. The serotonergic neurons send their projections to all regions of the brain and the spinal cord and some targets at the periphery. The raphe system is tonically active, while its activity can be modulated by different factors leading to the changes in serotonin release.

Serotonin mediates its responses via the superfamily of the 5-HT receptors, which contains 14 structurally and pharmacologically different receptor subtypes. The superfamily is now subdivided into 7 families (Fig. 1.3). Except the $5\text{-}HT_3$ receptor, which is the transmitter-gated Na^+/K^+ channel, all other 5-HT receptors belong to the class of the G-protein coupled receptors.

Figure 1.3. Phylogenetic Tree of the 5-HT Receptor Family. The superfamily is subdivided into 7 families. Except the 5-HT_3 receptor, which is the transmitter-gated Na^+/K^+ channel, all other 5-HT receptors belong to the class of G-protein coupled receptors. AC- adenylate cyclase, PLC- phospholipase C, IC- ion channel, „+"-activation, „-" - inhibition.



The **5-HT_1 receptor family** includes 5 subtypes, from 5-HT_1 A to F, that are encoded by different genes sharing 40 to 60 % sequence homology. All 5-HT_1 receptors are found to couple with pertussis-toxin sensitive $G_{i/0}$ proteins and to inhibit the adenylate cyclase activity (Albert et al., 1996; Boess and Martin, 1994; Saudou and Hen, 1994). The members of this family are broadly expressed in the limbic areas of the brain, particularly

in the hippocampus (5-HT_{1A}, 5-HT_{1D}, 5-$HT_{1E,}$ and 5-HT_{1F}), the raphe nuclei (5-HT_{1A}, 5-$HT_{1D,}$ and 5-HT_{1F}), the basal ganglia (5-$HT_{1B,}$ 5-$HT_{1D,}$ and 5-HT_{1E}) and the spinal cord (5-HT_{1D}). The 5-HT_1 receptors can be localized both post- and pre-synaptically, thus regulating the release of the 5-HT or of the other neurotransmitters. At the physiological level, some of the 5-HT_1 receptor agonists provoke the hyperphagia, activate the sexual behaviour, administrate the anxiolytic and the antidepressant action and increase the locomotion.

The **5-HT_2 receptor family** is composed of three receptor isoforms, 5-HT_2 A, B and C, that are structurally distinct from the other 5-HT receptors (Humphrey et al., 1993). All these receptors are coupled positively to the phospholipase C and mobilise the intracellular calcium (Barnes and Sharp, 1999) via the heterotrimeric $G_{q/11}$ proteins. The 5-HT_{2A} receptors are distributed post-synaptically in the forebrain (neocortex, enthorhinal and pyriform cortex), the caudate nuclei, the nucleus accumbens, the olfactory tubercle, and the hippocampus (Barnes and Sharp, 1999). Outside of the CNS, expression of the 5-HT_{2A} receptor was detected in the lung, the heart and the spleen. The 5-HT_{2B} receptor is expressed in the stomach fundus where it mediates the contraction (Barnes and Sharp, 1999). The 5-HT_{2C} receptor is expressed in the choroid plexus, the substantia nigra, the globus pallidus, the cerebral cortex and the olfactory tubercle. Administration of the 5-HT_{2C} receptor agonists influences locomotion, body temperature, feeding behaviour and hormone secretion. Also agonists of the 5-HT_{2C} receptor have anxiogenic effects (Boess and Martin, 1994).

The **5-HT_3 receptor** is the ligand-gated K^+/Na^+ channel. It is the only monoamine receptor to be associated with the fast synaptic transmission in the brain (Barnes and Sharp, 1999). Two homologous receptor subunits, 5-HT_{3A} and 5-$HT_{3B,}$ are essential for

the formation of the functional channel. In the brain, it is concentrated in the dorsal vagal complex of the brainstem. It is also detected in the hippocampus, the amygdala and the superficial layers of the cerebellar cortex. It is predominantly expressed in the GABAergic interneurons, where it plays an activating role, thus providing an indirect inhibition of pyramidal neurones. Report on the 5-HT_3 receptor knock-out mice indicates that the behavioural response to certain forms of pain was reduced in these animals (Guy et al., 1997).

The **5-HT_4 receptor subfamily** consists of the seven splice variants of the same gene differing in the sequence encoding for the cytoplasmic C-termini (Barnes and Sharp, 1999). All 5-HT_4 receptors mediate stimulation of adenylate cyclase via the stimulatory G_s proteins (Bockaert et al., 1990; Dumuis et al., 1988a). The 5-HT_{4b} receptor is also shown to couple with the $G_{i/0}$ proteins. In addition, the 5-HT_{4a} receptor has been shown to interact with the G_{13} proteins leading to the RhoA-mediated activation of gene transcription, neurite retraction and cell rounding (Ponimaskin et al., 2002b). 5-HT_4 receptors are localized in the collicular neurons, the basal ganglia, the hippocampus, the olfactory tubercle, the limbic structures and the pre-Bötzinger respiratory complex. Outside of the CNS, the 5-HT_4 receptors are found in the gastrointestinal tract, in the adrenal glands, in the myocardium and in the bladder. Functionally, the 5-HT_4 receptors contribute to the facilitation of the cognitive performances (Barnes and Sharp, 1999) and to the regulation of the respiratory activity (Manzke et al., 2003).

The **5-HT_5 receptor** family consists of two members, 5-HT_{5A} and 5-HT_{5B}. The 5-HT_{5A} receptor has been identified in mouse, rat and human. The 5-HT_{5B} receptor is also expressed in the mouse and rat, but not in the human where the coding sequence is interrupted by stop codons (Nelson, 2004). The 5-HT_{5A} receptor has been demonstrated to

couple to the $G_{i/o}$ proteins and to inhibit adenylate cyclase activity (Hurley et al., 1998). Both receptors are essentially limited in the distribution to the central nervous system, except that the 5-HT_{5A} receptor has also been found on neurons and neuron-like cells of the carotid body. Nothing is known about the role of the 5-HT_{5B} receptor in vivo. Recently, a mouse line has been developed where the 5-HT_{5A} receptor coding gene was knocked out and these animals have been shown to have a diminished increase in the LSD-induced locomotion (Nelson, 2004).

The **5-HT_6 receptor** is coupled positively to adenylate cyclase via the G_s proteins (Sebben et al., 1994). This receptor is shown to be expressed mainly in the CNS, although low levels of the receptor have been detected in the stomach and the adrenal glands. In the brain, the high levels of the 5-HT_6 receptor are present in the striatum, the olfactory tubercles, the nucleus accumbens and the hippocampus, being predominantly post-synaptic to the 5-HT neurones (Barnes and Sharp, 1999). The 5-HT_6 receptor appears to regulate the glutamatergic and the cholinergic neuronal activity. Recent data suggests that it may be involved in the regulation of cognition, feeding and, possibly, affective state and seizures (Woolley et al., 2004).

The **5-HT_7 receptors** are encoded by a single gene and the gene transcript undergoes an alternative splicing leading to the generation of at least four different isoforms. The 5-HT_7 receptors couple to the proteins of the G_s family, which leads to the activation of adenylate cyclase and increase in cAMP formation (Barnes and Sharp, 1999). In addition, the 5-HT_{7a} receptor stimulates the G_{12} proteins, which leads to the activation of small GTPases of the Rho family and to the stimulation of the transcriptional factor SRE (E. Kvachnina et. al., unpublished data). The 5-HT_7 receptors are expressed in the hypothalamus, the thalamus, the hippocampus and the cortex (Heidmann et al., 1998).

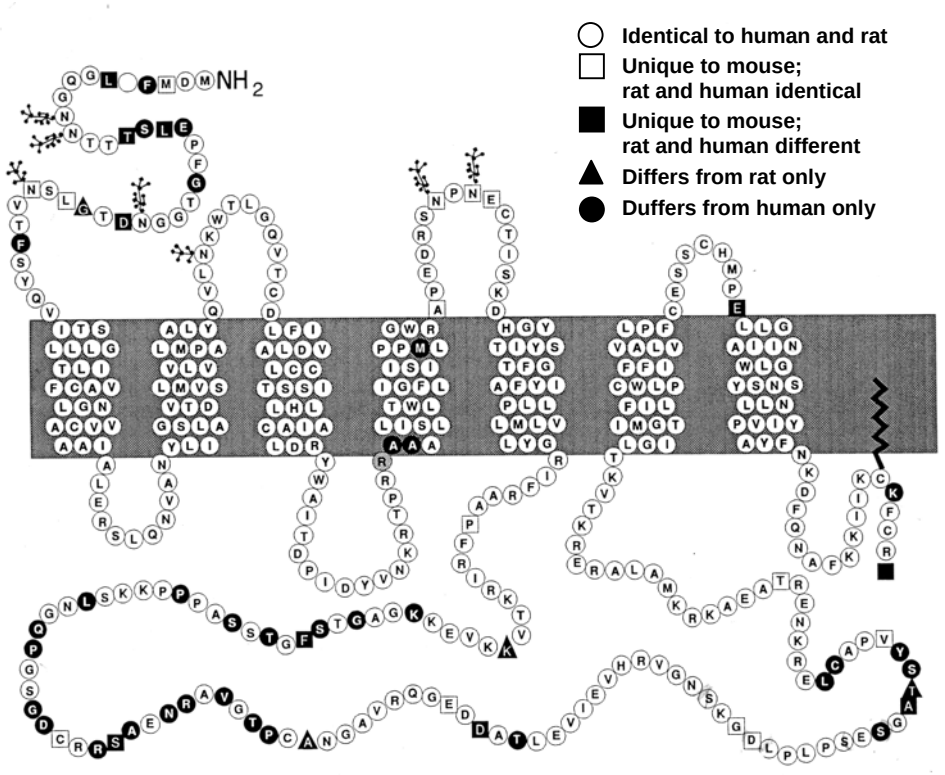
At the periphery, the 5-HT_7 receptors were found in smooth muscle cells of the blood vessels and in the gastrointestinal tract, where they mediate muscle relaxation (Thomas and Hagan, 2004). The 5-$HT_{(7)}$ receptors play an important role in control of both the circadian rhythms and the sleep (Thomas and Hagan, 2004).

1.4 The 5-HT_{1A} Receptor

The 5-HT_{1A} receptor (Fig.1.4) is most extensively characterized among the other 5-HT receptors. It was found to be involved in a number of physiological and behavioral effects, such as regulation of mood (Overstreet et al., 2003; Sibille and Hen, 2001), neuroendocrine responses (Burnet et al., 1996; Fletcher et al., 1996), body temperature (Overstreet, 2002), sleep states (Bjorvatn and Ursin, 1998), neurogenesis (Radley and Jacobs, 2002), cardiovascular system (Saxena and Villalon, 1990) and respiratory activity (Manzke et al., 2003; Richter et al., 2003). The 5-HT_{1A} receptor ligands are widely used in the treatment of the anxiety and the depression disorders (Dompert et al., 1985; Peroutka, 1985; Tunnicliff, 1991), and the characterization of the 5-HT_{1A} receptor knockout mice has supported the hypothesis that the receptor plays a role in those states (Parks et al., 1998). It has been suggested that the 5-HT_{1A} receptor ligands may have therapeutic utility in drug addiction (Cheeta et al., 2001), Alzheimer's dementia (Schechter et al., 2002) and some negative symptoms of schizophrenia (Meltzer, 1999). Indeed, the selective 5-HT_{1A} receptor blockade has been shown to enhance the signaling within the neuronal circuits involved in cognitive processes, thereby suggesting a novel therapeutic approach for the treatment of the cognitive disorders (Schechter et al., 2002).

The 5-HT_{1A} autoreceptors have been suggested to play an important role in the downregulation of firing of the serotonergic neurons in the raphe system. The delay in the onset of the therapeutic benefit, observed after the antidepressant treatment with 5-HT_{1A} agonists (e. g. bispirone) and selective serotonin reuptake inhibitors (SSRI) has been attributed to the slowly developing adaptive changes in the 5-HT_{1A} autoreceptors (Briley and Moret, 1993; Lanfumey and Hamon, 2004). On the other hand, recent studies using the selective regional rescue of the receptor in the 5-HT_{1A} knockout mice led to the conclusion that the postsynaptic 5-HT_{1A} receptors also play a role in the anxiety-like behavior (Overstreet et al., 2003).

Fig. 1.4. Schematic Representation of the Murine 5-HT_{1A} Receptor. The model is based on the hydropathisity analysis and the structural evidences from the other GPCR. Putative N-linked glycosylation sites are indicated by the small arboreous symbols. Putative palmitoylation is shown by the zigzag line (Charest et. al. 1993).



The 5-HT_{1A} receptor mediates its responses via the coupling to the pertussis-toxin sensitive heterotrimeric G-proteins of the $G_{i/o}$ families (Albert, 1994; Barnes and Sharp, 1999; Raymond et al., 1999), with the affinity decreasing in the following order: $G\alpha_{i3}>G\alpha_{i2}>G\alpha_{i1}>G\alpha_{0}>G\alpha_{z}$ (Lanfumey and Hamon, 2004). The coupling efficiency of the 5-HT_{1A} receptor to a given G-protein may be influenced by the type of the receptor agonist (Raymond et al., 1993). In addition, regional differences in the G-protein coupling to the 5-HT_{1A} receptor have been recently demonstrated (Lanfumey and Hamon, 2004). Thus, the 5-HT_{1A} receptor is coupled preferentially to the $G\alpha_{0}$ in the hippocampus, to the $G\alpha_{0}$ and the $G\alpha_{i3}$ in the frontal cortex, to the $G\alpha_{i3}$ in the dorsal raphe nucleus, and to the $G\alpha_{i1}$, the $G\alpha_{i3}$ and the $G\alpha_{z}$ in the hypothalamus. The receptor-mediated activation of the $G\alpha_{i}$-subunits results in the inhibition of the adenylate cyclase (AC) and a subsequent decrease of the cAMP levels. This effect was monitored in the hippocampal neurons (De Vivo and Maayani, 1986; Dumuis et al., 1988b) as well as in the different cell lines (Fargin et al., 1989; Liu and Albert, 1991; Nebigil et al., 1995). Analysis of G-protein specificity for the 5-HT_{1A} receptor mediated AC inhibition revealed an unexpected complexity. Antisense depletion of the different subtypes of the $G\alpha_{i}$-subunit revealed that the removal of the $G\alpha_{i1}$ eliminated the 5-HT_{1A} receptor induced inhibition of a basal cAMP level, whereas the depletion of the $G\alpha_{i2}$ and the $G\alpha_{i3}$ blocked the 5-HT_{1A} receptor action on a G_{s}-activated adenylate cyclase (Liu et al., 1999). In addition, positive coupling of the recombinant 5-HT_{1A} receptor to the cAMP production has been shown in the cells expressing the AC2 isoform of the adenylate cyclase (Albert et al., 1999). Besides the effects mediated by the $G\alpha_{i/o}$ subunits, activation of the 5-HT_{1A} receptor leads to the $G\beta\gamma$-

mediated activation of the inwardly rectifying K^+ channels and the inhibition of the L-type Ca^{2+} channels in hippocampal neurons (Andrade et al., 1986; Clarke et al., 1996; Zgombick et al., 1989), dorsal raphe nucleus neurons (Clarke et al., 1996) and atrial myocytes (Karschin et al., 1991). In recombinant cell lines, the 5-HT_{1A} receptor evokes the Gβγ-mediated stimulation of the phosphatidilinositol-specific phospholipase C (PI-PLC) (Raymond et al., 1991). In addition, the 5-HT_{1A} receptor can activate the mitogen–activated protein kinase Erk 1/2. By the modulation of the Erk 1/2 activity the 5-HT_{1A} receptor may be involved in the regulation of cell proliferation (Raymond et al., 2001), neurogenesis (Radley and Jacobs, 2002) and neuroprotection (Adayev et al., 2003).

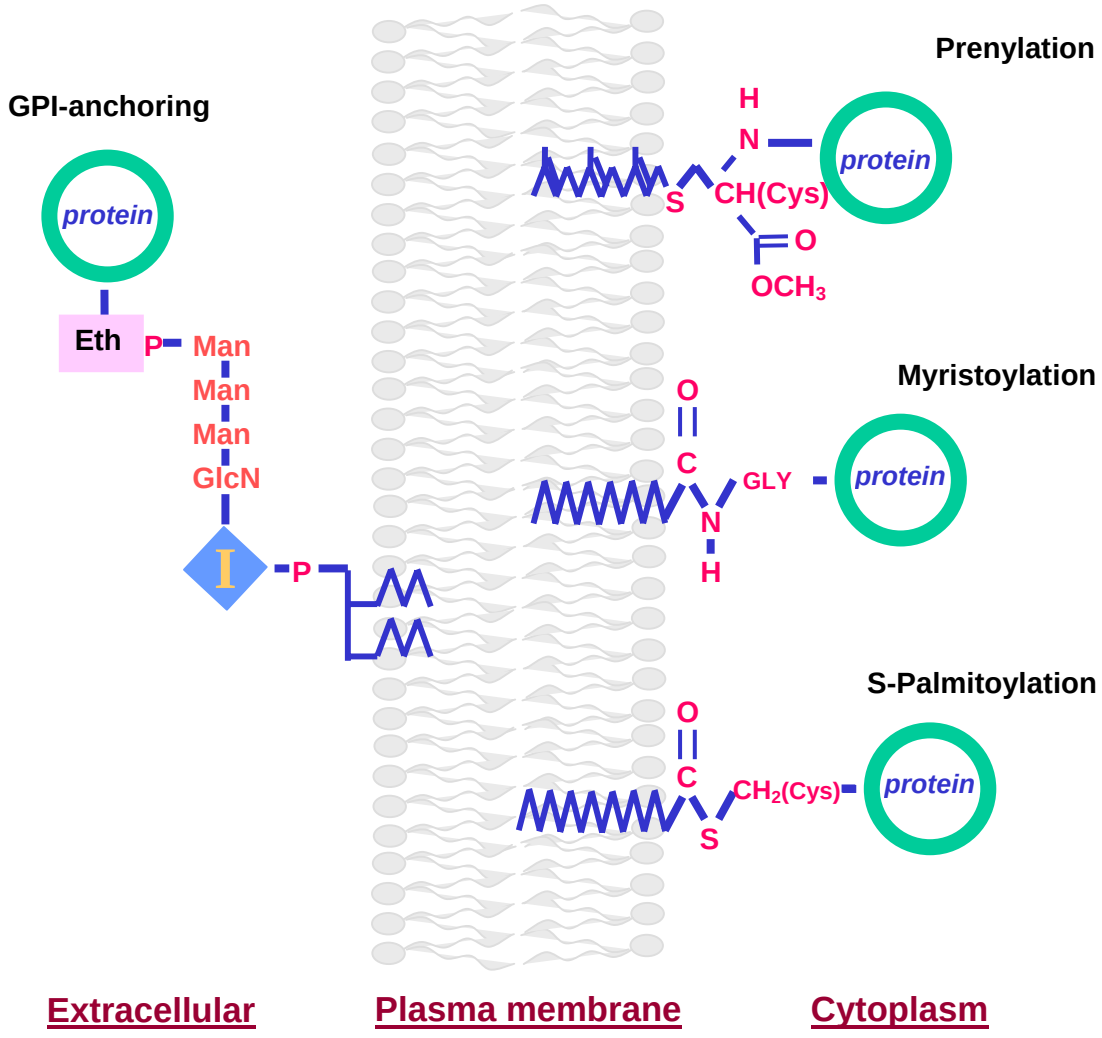
1.5 Hydrophobic Modifications of the Proteins

Proteins are often subjected to the different co- and post-translational modifications, such as the phosphorylation, the glycosylation or the lipidation. These modifications produce an additional level of complexity that is often involved in the regulation of the protein functions. The covalent attachment of the lipid moieties represents an essential modification found in many proteins. Three types of lipid modifications are recognized so far: GPI-anchoring, prenylation and acylation (Fig 1.5). The later includes N-myristoylation and S-palmitoylation (Bhatnagar and Gordon, 1997; Casey and Seabra, 1996; Resh, 1999).

The **GPI-anchoring** means a post-translational attachment of the glycophosphatidilinositol moieties to a target protein. The core structure of the GPI-anchor consists of ethanolamine phosphate, trimannoside, glucosamine and inositol phospholipid. The GPI anchor is attached to the carboxyl terminus of proteins by the

ethanolamine head. The process of GPI attachment is catalyzed by the GPI-transamidase complex (Ikezawa, 2002) .

Figure 1.5. Major Lipid Modifications of Proteins. Gly-glycine, Cys-cysteine, Eth-ethanolamine, P-phosphate, Man-mannose, GlcN-glucosamine, I-inositol. Prenylation is an irreversible post-translational modification of the protein by unsaturated fatty acids via the stable thioether bond. Myristoylation is a stable co-translational modification of the N-terminal glycine by the saturated myristic acid. Reversible S-palmitoylation occurs at the cysteine residues via the thioester-type bond. The GPI-anchor consists of the phosphatidil-ethanolamine, trimannoside, glucosamine and inositol phospholipid and serves for the attachment of proteins to the outer leaflet of the plasma membrane.



Prenylation is a lipid modification involving covalent attachment of either farnesyl (15-carbon) or geranyl-geranyl (20-carbon) isoprenoids via the thioether linkages to the cysteine residues located near to the C terminus of the target proteins. The prenylation is catalyzed by the specific enzymes named protein prenyltransferases, that are classified in the two functional classes: the CAAX prenyltransferases and the protein geranylgeranyltransferase type I. The prenylation is often required for the proper function of the modified proteins, either as a mediator of the membrane association, as a transport signal, or as a determinant for the specific protein-protein interactions (Casey and Seabra, 1996).

N-myristoylation occurs co-translationally by addition of the 14-carbon saturated myristic fatty acid to the N-terminal glycine residue localized within the consensus sequence. The myristoylation occurs via the amide bound. It is catalyzed by the specific enzyme N-myristoyl-transferase (Raju and Sharma, 1999). Functionally, myristoylation is involved in the membrane anchoring of the target proteins (Resh, 1999).

S-palmitoylation is a reversible attachment of palmitate or other saturated long chain fatty acids to the target proteins. The attachment of the fatty acids occurs at the cysteine residues via the thioester linkage. The palmitoylation is unique among the lipid modifications as it can be reversible and adjustable. Among the cellular palmitoylated proteins, polypeptides involved in the signal transduction (e.g. GPCRs, α-subunits of G-proteins, Ras-protein, endothelial nitric oxide synthase, adenylate cyclase, phospholypase C and non-receptor tyrosine kinases) are often targets for this dynamic modification (Bijlmakers and Marsh, 2003; Bouvier et al., 1995b; Dunphy and Linder, 1998). Meanwhile it is widely accepted that the repeated cycles of palmitoylation and

depalmitoylation can be critically involved in the regulation of the signaling processes (Cramer et al., 2001; Mumby, 1997; Ross, 1995).

1.6. Mechanisms of the Protein S-Palmitoylation

Sites of the palmitoylation. Comparison of the amino acid sequences of the palmitoylated proteins does not reveal any consensus sequence for this modification. Types of the proteins that undergo palmitoylation are rather diverse and include both integral and membrane associated proteins (Linder and Deschenes, 2003). Palmitoylation often takes place at the domains that are situated close to the membranes. For example. integral membrane proteins, such as the GPCRs, the tetraspanins and the viral glycoproteins have been shown to be palmitoylated at cysteine residues located in the close vicinity of the membrane. Examples of the dependence of palmitoylation on the proximity to the membrane are given by dually lipidated proteins, such as the farnezylated and palmitoylated Ras-proteins, the myristoylated and palmitoylated G_i proteins, the Fyn kinase and the endothelial nitric oxide synthase. It has been suggested that first modification (farnesylation or myristoylation) provides transient association of protein with the membrane which allows for consequent palmitoylation.

Another large group of palmitoylated proteins includes the membrane-associated proteins that do not reveal any means of the membrane association except the palmitoylation itself. This group includes the postsynaptic density protein PSD95, the $G\alpha_q$ subunit and some of the mitochondrial enzymes. Palmitoylation of these proteins seems to be independent on their position relative to the membrane, and thus might involve different mechanisms.

Non-enzymatic palmitoylation. It has been proposed by several research groups that the palmitoylation might occur non-enzymatically, and the palmitoylated state of proteins can be regulated by the enzymatic depalmitoylation step (Bano et al., 1998; Bharadwaj and Bizzozero, 1995; Bizzozero et al., 2001; Duncan and Gilman, 1996); (Duncan and Gilman, 1996; O'Brien et al., 1987). However, non-enzymatic palmitoylation has been shown only in *in vitro* assays and needs a non-physiologically high concentration of palmitoyl-CoA (Linder and Deschenes, 2003).

Enzymatic palmitoylation. The majority of data on the protein palmitoylation suggests that both palmitoylation and depalmitoylation are catalysed by the enzymatic reactions. Palmitoyl Acyl Transferase activity (PAT) has been detected in the membrane fractions derived from a variety of cell types (Berger and Schmidt, 1984; Mack et al., 1987). The PAT activity has been also detected in the membrane preparations enriched in the ER, the Golgi and the plasma membrane (Berthiaume and Resh, 1995; Das et al., 1997; Dunphy et al., 1996; Gutierrez and Magee, 1991; Liu et al., 1996; Ueno and Suzuki, 1997). Given the diverse nature of the palmitoylated proteins, it would not be surprising if multiple protein acyltransferases would be found in different cell types or even within one cell type.

Genetic studies performed in the yeast provided a new insight into the nature of the PAT enzymes. Two types of the PATs were isolated from the yeast using the genetic screen (Roth et al., 2002; Zhao et al., 2002). Both of the enzymes reveal the cysteine rich domains (CRD) containing the conserved DHHC (Asp-His-His-Cys) motifs, that are shown to be critical for their PAT activity. The isolated Erf2/Erf4 complex has been shown to catalyse protein palmitoylation on the cysteine residues adjacent to the sites of farnezylation (yeast Ras2 protein) (Dong et al., 2003). Another yeast protein with the PAT

activity, the AKR1, catalyses the palmitoylation of the casein kinase Yck2p via an ATP-dependent mechanism (Roth et al., 2002). Interestingly, the AKR1 protein seems to be capable to palmitoylated cysteines in the absence of any adjacent lipid modification. This implies that the AKR1 and the Erf2/Erf4 PATs recognise distinct substrates. Such different substrate preferences of the two isolated PAT enzymes correlate with the data on structure of the palmitoylated proteins described above.

Genes encoding for the DHHC-CRD containing proteins are found in all eukaryotic genomes examined to date (Linder and Deschenes, 2003). Thus mouse and human genomes appear to have approximately 23 DHHC-CRD genes, some of which are currently under investigation.

Enzymatic depalmitoylation. The fact that the rate of the palmitate turnover often exceeds that of the protein itself, suggests that the depalmitoylation is an enzymatic process. Two kinds of the palmitoylthioesterases, the one lysosomal (PPT1 and PPT2) and the other cytoplasmic (APT1) enzymes have been identified and characterised so far (Linder and Deschenes, 2003).

The protein palmitoyl thioesterases PPT1 and PPT2 are localized exclusively within the lysosomes (Camp et al., 1994; Soyombo et al., 1999). They play an important role in the catabolism of palmitoylated proteins. Deficiency in the PPT1 enzyme was shown to be associated with the neurodegenerative disorder, infantile neuronal ceroid lipofuscinosis (INCL). Several cell types of the INCL patients reveal an abnormal accumulation of the lipidated thioesters derived from the acylated proteins (Lu et al., 1996).

The cytoplasmic acyl protein thioesterase APT1 has been proposed to be involved in the regulation of the palmitate turnover on many cytosolic and membrane proteins (Duncan and Gilman, 1998). The purified APT enzyme can cleave both thio- and

oxyesters. The APT 1 was shown to regulate the palmitate turnover on the $G\alpha_s$ protein, the endothelial nitric oxide synthase and some of the viral proteins (Linder and Deschenes, 2003; Veit and Schmidt, 2001). It would be interesting to determine whether the APT1 can deacylate the integral membrane proteins such as GPCRs.

1.7 Palmitoylation of the G-protein Coupled Receptors

As was mentioned above, most of the GPCRs contain conservative cystein residues at the cytoplasmic C-terminus, which represent putative palmitoylation sites. In many cases palmitoylation of the GPCR was confirmed experimentally by point mutagenesis and truncation analysis (Qanbar and Bouvier, 2003).

Palmitoylation of several GPCRs has been shown to be a dynamic and agonist-dependent modification. For example, treatment with agonists increased incorporation of the palmitate in the β_2-adrenergic (Mouillac et al., 1992), the 5-HT_4 (Ponimaskin et al., 2001), the muscarinic acetylcholine m2 (Hayashi and Haga, 1997) and the α2a adrenergic receptors (Kennedy and Limbird, 1994). The increased palmitate incorporation was interpreted as a facilitated turnover rate of the palmitate on the activated receptors. On the contrary, incorporation of the palmitate in the vasopressin V2 receptor was decreased upon the agonist stimulation (Sadeghi et al., 1997). For some receptors, such as the human A1 adenosine receptor, no dependence of the palmitate incorporation on the receptor activation has been observed (Gao et al., 1999).

Variable roles have been assigned to the palmitoylation of GPCRs (Qanbar and Bouvier, 2003). The fact that different effects were observed for various GPCRs upon the mutation of the palmitoylated cysteines could reflect individual characteristics of the

receptor studied. The functional roles played by the palmitoylation in the curriculum of the different GPCRs are summarized below.

Palmitoylation and cell surface expression of the receptor. Some studies revealed that the GPCR palmitoylation may be involved in processing and membrane targeting of the receptors. Initial palmitoylation of the GPCRs occurs either in the ER-Golgi intermediate or in the early Golgi compartments (Bradbury et al., 1997) and appears to be important for the expression of the functional receptors on the cell surface. Intracellular trapping of the receptors upon the removal of the palmitoylation sites was demonstrated for the rhodopsin (Karnik et al., 1993), the lutropin/CG receptor (Zhu et al., 1995), the canine H2 histamine receptor (Fukushima et al., 2001) and the CCR5 cytokine receptor (Percherancier et al., 2001). Moreover, the lack of the palmitoylated cysteines can be accompanied by the enhanced receptor degradation, as was demonstrated for the adenosine A1 receptor (Gao et al., 1999).

Coupling to the G-proteins and the downstream effectors. Replacement of the palmitoylation sites in several GPCRs has also been shown to affect the downstream signaling. For example, replacement of a palmitoylation site of the acetylcholine m2 receptor reduced its ability to couple with the $G\alpha_0$ and the $G\alpha_i$ proteins (Hayashi and Haga, 1997). The nonpalmitoylated human somatostatin receptor type 5 displayed the reduced coupling to an adenylate cyclase inhibition (Hukovic et al., 1998), whereas mutation of a palmitoylation site in the β2-adrenergic receptor impaired its interaction with the $G\alpha_s$ protein (Moffett et al., 1993; O'Dowd et al., 1989).

Sometimes, palmitoylation plays various roles in the different signaling pathways activated by the same receptor. For example, the nonpalmitoylated mutant of the human

endothelin A receptor was impaired in coupling with $G\alpha_i$ and $G\alpha_q$ proteins, while its interaction with the $G\alpha_0$ protein was not affected (Doi et al., 1999). Different functions in the modulation of the downstream effectors were also attributed to the palmitoylated cysteines of the endothelin B (ETB) receptor (Okamoto et al., 1998b). While the nonpalmitoylated mutant failed to stimulate the phospholipase C via the $G\alpha_q$ and to inhibit the adenylate cyclase via the $G\alpha_i$, the receptor in which only one of three modified cysteines was mutated retained the ability to stimulate the phospholipase C, but not to inhibit the adenylate cyclase. On the contrary, in case of the 5-HT_4 receptor, the mutation of one palmitoylation site increased the agonist-independent coupling of the receptor with the G_s protein and the adenylate cyclase.

In contrast to the above data, lack of palmitoylation does not significantly influence coupling of the α_{2a} adrenergic receptor with the G-proteins (Kennedy and Limbird, 1993). Similar results were obtained for the lutropin/CG receptor (Kawate et al., 1997), the dopamine D_1 receptor (Jin et al., 1997), the human A_1 adenosine receptor (Gao et al., 1999) and the human thyrotropin receptor (Tanaka et al., 1998).

Phosphorylation and desensitization. Desensitization of GPCRs is triggered by receptor activation and leads to the uncoupling of the receptor from the signaling. The cascade of events that leads to the desensitization is initiated by phosphorylation of the receptor by the kinases, followed by interaction of the receptor with the β-arrestin and internalization. Palmitoylation of the GPCRs was proposed to play an important role in the regulation of receptor desensitization. Thus, palmitoylation-deficient mutant of the β2-adrenergic receptor was shown to be hyperphosphorylated at the basal level, and its phosphorylation did not increase upon the agonist stimulation (Moffett et al., 1993).

Taken together with the fact that agonist stimulation promoted an increase of a palmitate turnover (Mouillac et al., 1992), palmitoylation of the b2-adrenergic receptor could be considered as a molecular switch regulating the desensitization of the receptor by changing the accessibility of the phosphorylation sites. Similar conclusions appeared from structural analysis of the rat bradikynin B2 receptor, which demonstrated the mutual exclusion of palmitoylation and phosporylation at sites located close to each other (Soskic et al., 1999).

In contrary, the acylation-deficient V_{1a} vasopressin (Hawtin et al., 2001) and the CCR5 chemokine (Kraft et al., 2001) receptors have been shown to possess decreased phosphorylation at the basal and the agonist-activated states. The decrease of phosphorylation has been accompanied by the reduced internalization, arguing for a coordinated regulation of the receptor palmitoylation, the phosphorylation and the endocytosis.

In several cases palmitoylation has been found to be involved in the internalization process. For the CCR5 chemokine (Kraft et al., 2001), the thyrotropin releasing hormone (Groarke et al., 2001) and the human somatostatin type 5 receptors (Hukovic et al., 1998), mutation of the palmitoylation sites was found to decrease the rate of the agonist-promoted internalization. In case of the thyrotropin releasing hormone receptor, palmitoylation has been shown to be critical for its interaction with β-arrestin (Groarke et al., 2001). In contrast, mutation of the palmitoylation sites of the lutropin/CG receptor lead to the dramatic increase of the internalization rate (Kawate et al., 1997).

Following the internalization, many of the GPCRs are subjected to downregulation, and palmitoylation has been shown to play a role in this process. Replacement of the palmitoylation sites of the lutropin/CG receptor (Bradbury et al., 1997) and the H_2

histamine receptor (Fukushima et al., 2001) facilitated their downregulation. In contrast, mutation of the palmitoylation site of the α_{A2} adrenergic receptor completely abolished the receptor downregulation even upon prolonged agonist stimulation (Eason et al., 1994).

1.8 GPCRs in Lipid Rafts and Caveolae

"Lipid rafts" represent cell membrane domains enriched in specific lipids and proteins. They are characterised by a high glycosphingolipid and cholesterol content in the outer leaflet of the lipid bilayer that gives them a gel-like liquid-ordered (L_o) structure (Brown and London, 1998). The caveolae that are the invaginated microdomains of the plasma membrane (Yamada, 1955), enriched in specific caveolae proteins (caveolin 1, 2 and 3), glycosphingolipids and cholesterol (Chini and Parenti, 2004), are considered to be a subfamily of the lipid rafts The lipid rafts and the caveolae are resistant to the low-temperature solublization by non-ionic detergents (Brown and London, 1998), and therefore are often termed "detergent-resistant membrane subdomains" (DRM). This property allows for their biochemical separation due to the differential flotation in the density gradients.

The DRM were found to be involved in the regulation of numerous cell functions, including the intracellular sorting of proteins and lipids (Sprong et al., 2001), the establishment of cell polarity (Manes et al., 2003), the vesicular transport processes (Conner and Schmid, 2003; Johannes and Lamaze, 2002; Nabi and Le, 2003) and the cholesterol homeostasis (Ikonen and Parton, 2000). Several studies also demonstrated an important role of the DRM in the fine tuning of the signaling processes (Simons and Ikonen, 1997). A number of the receptor tyrosine kinases, the G-protein coupled

receptors, the G-proteins, the kinases and the phosphatases have been found to be located in the DRMs (Foster et al., 2003). Therefore it is believed, that the lipid rafts can assist to the efficient signal transmission by organising the interacting elements of one signaling system in the close proximity to each other.

Several GPCRs were shown to be enriched or almost exclusively located in the lipid rafts or the caveolae. For example, more than 90% of the gonadotropin-releasing hormone (GnRH) receptor are localized in lipid rafts (Navratil et al., 2003). Some receptors are represented in the DRMs only by a small fraction. For example, the fraction of the oxytocin receptor (OTR) in the lipid rafts comprises less than 10% of total amount of the receptor on the membrane (Gimpl and Fahrenholz, 2000). Furthermore, some GPCRs has been shown to translocate inside or outside of the lipid rafts/caveolae during activation/deactivation (Chini and Parenti, 2004). The DRMs may be involved in trafficking and stabilisation of some GPCRs on the plasma membrane (Chini and Parenti, 2004) and in coupling of the receptors to the certain signaling pathways (Yamaguchi et al., 2003). In addition, lipid rafts and caveolae were shown to take part in a clathrin-independent endocytosis of some GPCRs.

The targeting signal(s) responsible for the localization of GPCRs in the lipid rafts and the caveolae are generally unknown. One of the hypotheses proposes that GPCRs are transported to DRM due to the interaction of extracellular parts of the receptor with raft gangliosides, analogous to such interaction in a case of the EGF receptor. Other researchers believe that an interaction of transmembrane domains with the lipid bilayer, especially cholesterol, may dictate the microdomain localization of GPCR. In addition, interactions with some proteins could also be responsible for the targeting of GPCRs to the lipid rafts or caveolae. For example, several caveolae-localized GPCRs, such as the β-

adrenergic, the endothelin A and the muscarinic m2 receptors, are known to interact with caveolin 1 (Chini and Parenti, 2004).

One of the mechanisms for the protein targeting into the DRMs is their covalent modification by fatty acids, since the long-chain saturated fatty acids may pack well in the L_o ordered phase and increase the protein avidity for the sphingolipid/cholesterol-enriched domains (Melkonian et al., 1999; Moffett et al., 2000). A number of the palmitoylated proteins or the proteins modified by other lipids, such as the α-subunits of the heterotrimeric G-proteins, the non-receptor tyrosine kinases (NRTKs) and the endothelial nitric oxide synthase (eNOS), are localized mainly in the DRM (Okamoto et al., 1998a). It has also been demonstrated, that removal of the fatty acid modifications leads to the loss of the protein association with the lipid rafts and caveolae (Moffett et al., 2000; Shaul et al., 1996; Shenoy-Scaria et al., 1994; Song et al., 1997). For the GPCRs, the role of the fatty acylation for the DRM trafficking remains unclear, as many of the palmitoylated GPCRs are shown to be excluded from the lipid rafts.

2. AIM OF THIS WORK

The aim of the present work was to investigate the acylation of the 5-HT_{1A} receptor. The following aspects were studied:

1. Detection of possible hydrophobic modifications of the 5-HT_{1A} receptor
2. Analysis of the chemical nature of the acylation
3. Investigation of the dynamics of 5-HT_{1A} receptor acylation
4. Determination of the palmitoylation site(s)
5. Functional analysis of the 5-HT_{1A} receptor palmitoylation

During the experimental work we found that the palmitoylation of the 5-HT_{1A} receptor is an irreversible modification. This finding is unique among the multiple data published for the palmitoylated signaling proteins because usually this modification is dynamic. In addition, we found that the 5-HT_{1A} receptor palmitoylation is not modulated by the agonist stimulation. Using the acylation-deficient mutants we analysed the role of 5-HT_{1A} receptor palmitoylation in the receptor-mediated downstream signaling, including the coupling with the inhibitory G-proteins, the inhibition of stimulated cAMP formation and the stimulation of the mitogen-activated protein kinase. The analysis revealed that the palmitoylation of the 5-HT_{1A} receptor is critical for the receptor-mediated signal transduction. Finally, we analysed possible role of the palmitoylation in targeting of the 5-HT_{1A} receptor to the detergent-resistant plasma membrane subdomains.

3. MATERIALS AND METHODS

3.1. Materials Used

3.1.1. Chemicals

Applied Biosystems : *AmpliTaq Gold PCR Kit*

Amersham Biosciences : *ECL™ Western Blot Detection Reagents, Nitrocellulose membrane, Blocking reagent, 5-hydroxy[^{3}H] tryptamine trifluoroacetate (107 Ci/mmol).*

Calbiochem : *Cycloheximide*

Genomed: *Jetsorb Gel Extraktion Kit*

Corning: 20 μm PVDV membranes mounted in 96-well microplates

Hartmann Analytic GmbH: *[9,10-^{3}H] Palmitic acid (30-60 Ci/mmol).*

ICN: *Tran [^{35}S]-label methionine (1000Ci/mmol).*

Invitrogen: *Oligonucleotide primers, Cellfectin, Lipofectamin 2000, TC-100 medium, DMEM-Glutamax II medium, Genetizin, FCS (Fetal Calf Serum), Trypsin, S.O.C. Medium, YT medium.*

Kodak: *Kodak X-Omat AR film*

New England Biolabs: *Enzymes used in the molecular cloning.*

Nunc: *Cell culture plasticware*

PerkinElmer Life Sceince: *[^{35}S] GTPγS (1300 Ci/mmol.)*

Qiagen: *QIAGEN Plasmid DNA purification Maxi Kit*

Roth: *Ammoniumpersulfat, Ampicillin (Potassium salt), TEMED, Acrylamide, Bis-Acrylamide, 2-Merkapthoethanol, Glycin, X-gal, IPTG.*

Serva:, *Bromphenol blue*

Sigma: *5-Hydroxytryptamine, F-12 HAM nutritient mixture, 8-OH-DPAT, Protein A-Sepharose CL-4B beads, Gentamicin Sulfate, Kanamicine Sulfate, SDS, Ethidiumbromide, Penicillin/Streptomycin solution, PMSF, GDP, GTP, Bacto-Tryptone, Bacto-Yeast extract, Bacto-Agar, Protein Standard Kit* for the protein concentration measurements.

3.1.2. Antibodies

Anti-HA - mouse monoclonal and rabbit polyclonal antibodies **(Santa Cruz Biotechnology)** raised against a 9 amino acids hemagglutinine-tag (YPYDVPDYA).

Anti-GFP (Abcam) - rabbit polyclonal antibodies raised against a purified Green Fluorescent Protein (GFP).

Anti $G\alpha_{i3}$ (Santa Cruz Biotechnology) - affinity purified rabbit polyclonal antibodies raised against a peptide mapping at the carboxyl terminus of $G\alpha_{i3}$ of rat origin. The antibodies also recognise $G\alpha_{i1}$ and $G\alpha_{i2}$ subunits.

Anti $G\alpha_s$ (Santa Cruz Biotechnology) - affinity purified rabbit polyclonal antibodies raised against a peptide mapping within the amino terminal domain of the $G\alpha_s$ of human origin.

Anti-$G\alpha_{12}$ - rabbit polyclonal antibodies raised against the $G\alpha_{12}$ protein (Ponimaskin et al., 1998)

Anti-Gα_{13} (Santa Cruz Biotechnology) - rabbit polyclonal antibodies raised against the N-terminus of the murine Gα_{13} protein.

Anti-caveolin 1 (Santa Cruz Biotechnology) - rabbit polyclonal antibodies raised against the N-terminal peptide of caveolin 1.

Anti-CD71 (Santa Cruz Biotechnology) rabbit polyclonal antibodies raised against the extracellular domain of the human transferrin receptor (CD71).

p42/44 (New England Biolabs) – rabbit polyclonal antibodies raised against the mitogen-activated protein kinase Erk 1/2.

phospho-p42/44 (New England Biolabs) - mouse monoclonal antibodies, raised against the active form of the mitogen-activated protein kinase Erk 1/2 phosphorylated at the residues Threonine 202 and Tyrosine 204.

Peroxidase conjugated goat anti-mouse and anti-rabbit antibodies (Amersham Pharmacia Biotech).

3.1.3. Plasmids:

pcDNA3.1(-), pcDNA 3.1 (+) Myc/His (Invitrogen)

pFastBac (Invitrogen)

pEYFP (Clontech)

3.1.4. Oligonucleotides (Invitrogen):

HA-1A-Sense:

5´ - GGAGTGGTACCCACCAT GGATTACCCATACGACGTCCCAGACTACGC TATGGATATGTTCAGTCTTGGC – 3´

1A-Antisense:

5´ - CAGGGGGTACCTATTGAGTGAACAGGAAGGGTC – 3´

Cys 417 –Ser Forward

5’ - GAT CAT CAA GT**C** CAA GTT CTG -3’

Cys 417 –Ser Reverse

5’ - C AGA ACT TG**G** ACT TGA TGA TC – 3’

Cys 420 –Ser Forward

5’ - GTG CAA GTT CT**C** CCG CTG ATG – 3’

Cys 420 –Ser Reverse

5’ - CAT CAG CGG **G**AG AAC TTG CAC – 3’

Cys 417, 420- Ser Forward

5’ CAA GT**C** CAA GTT CT**C** CCG CTG - 3’

Cys 417, 420- Ser Reverse

5’ - CAG CGG **G**AG AAC TTG **G**AC TTG – 3’

Chimera Sense-KpnI:

5’ – ATTCCGGTACCGCGAGGGAGATCCCCTTG – 3’

Chimera Antisense WT KpnI:

5’ – ATCATGGTACCGGGCGGCAGAACTTGCAC -3’

Chimera Antisense DM- KpnI:

5’ – ATCATGGTACCGGGCGGGAGAACTTGGAC – 3’

3.1.5. Buffers:

Blocking solution: 5% (w/v) ECL blocking reagent in PBS-Tween

GTPγS buffer A: 50 mM Tris/HCl, pH 7.4, 2 mM EDTA, 100 mM NaCl, 3 mM $MgCl_2$ and 1 µM GDP

GTPγS buffer B: 50 mM Tris/HCl, pH 7.5, containing 20 mM $MgCl_2$, 150 mM NaCl, 0,5% NP-40, 200 µg/ml aprotinin, 100 µM GDP and 100 µM GTP

Hepes-EDTA: 20 mM Hepes, 1 mM EDTA, pH 8.0

Ligand binding buffer: 50 mM Tris (pH 7.7), 0.1 % ascorbic acid, 20 µM pargyline

Ligation buffer: 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP 25 µg/ml BSA, pH 7.5

NTEP: 0.5% NP-40, 150 mM NaCl, 50 mM Tris/HCl (pH 7.9), 5 mM EDTA, 10 mM Jodinacetamide, 1mM PMSF. glycine, 0.1% SDS, pH 8.4

P1 buffer: 50 mM Tris-HCl, 10 mM EDTA, 100 µg/ml RNAseA, pH 8.0

P2 buffer: 200 mM NaOH, 1% SDS (w/v)

P3 buffer: 3 M CH_3COONa, pH 5.0

PBS: 140 mM NaCl, 3mM KCl, 2mM KH_2PO_4, pH 7.4

PBS-Tween: PBS, 0.05 % (w/v) Tween 20

PCR buffer: 20 mM Tris/HCl, 50 mM KCl, pH 8.4

Protein electrophoresis separation buffer: 375 mM Tris-HCl, 3.5 mM SDS, pH 8.8

TE: 0.01 M Tris-HCl, 7.6 or 7.4, 1 mM Na_2EDTA, pH 8.0.

Protein electrophoresis stacking buffer: 125 mM Tris-HCl, 3.5 mM SDS, pH 6.8.

Protein loading buffer: 31 mM Tris/HCl, 10% Glycerin, 3% SDS, 0,05 % bromphenolblue pH 8.8

QBT buffer: 750 mM NaCl, 50 mM MOPS, 15 % isopropanol, 0.15 % triton X-100, pH 7.0

QC buffer: 1 M NaCl, 50 mM MOPS, 15 % isopropanol, pH 7.0

QF buffer: 1.25 M NaCl, 50 mM Tris-HCl, 15 % isopropanol, pH 8.5

Restriction buffer 1: 20 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.0

TAE: 40 mM Tris, 1 mM Na_2EDTA, 20 mM acetic acid, pH 8.0

TE: 10 mM Tris-HCl, 1mM EDTA, pH 8.0

TNE buffer: 25 mM Tris-Hcl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 10% sucrose, 1% Triton X-100, 1 mM PMSF, 10 μM leupeptin, 2 μg/ml aprotinin

Transfer buffer: 25 mM Tris/HCl, 192 mM glycine, 20% (v/v) methanol, pH 8.3

All chemicals were purchased from Roth, if not marked otherwise.

3.2. Recombinant DNA Procedures

The basic DNA procedures were performed as described by Sambrook et al. (Sambrook, 1989). The m5-HT_{1A} cDNA was kindly provided by Dr. Paul R. Albert (Ottawa, Canada).

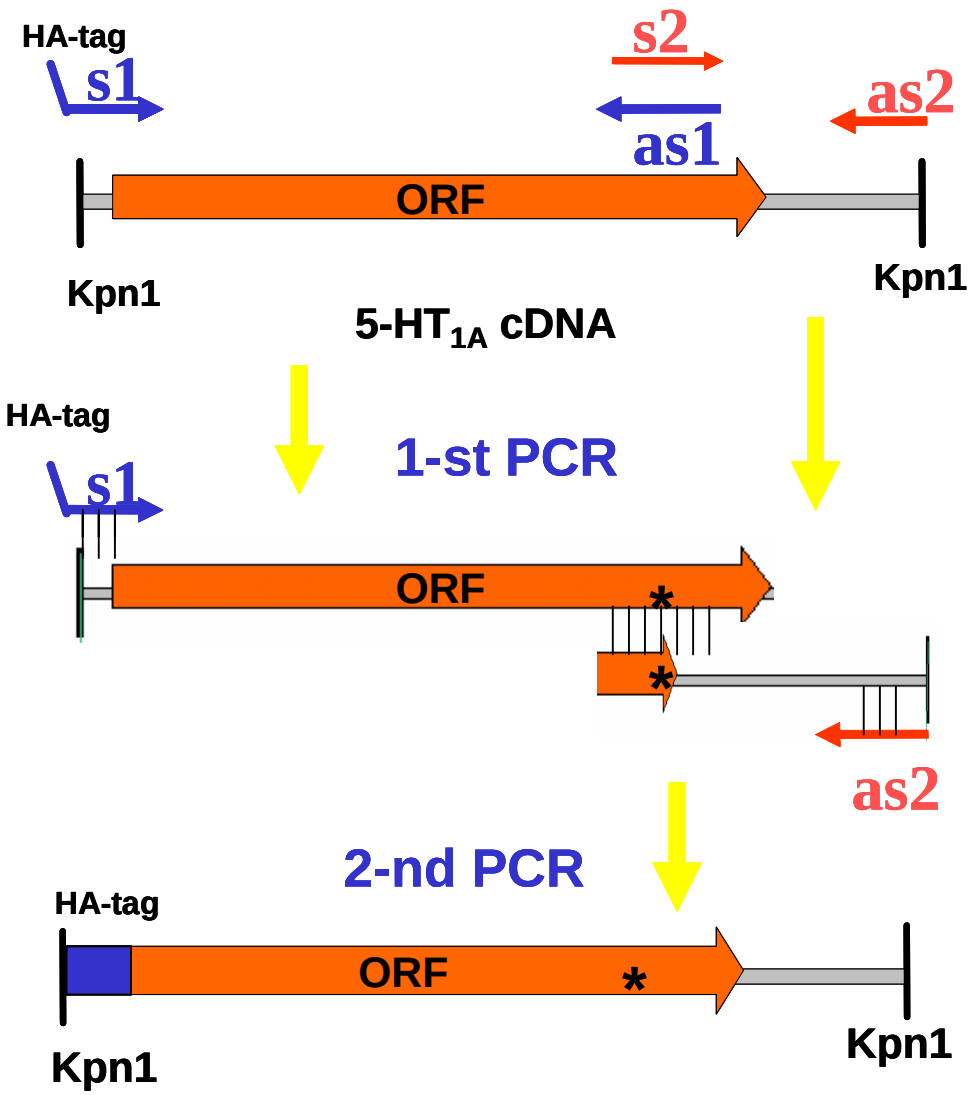
3.2.1. PCR, Site-Directed Mutagenesis and Cloning

The PCR primers were designed using the Vector NTI software (InforMaxInc, 1994-2002). The m5-HT_{1A} cDNA was amplified with the specific primers *HA-1A-Sense* and *1A-Antisense* to create the 9 amino acids hemaglutinine-tag (YPYDVPDYA) at the N-terminus of the receptor. The PCR fragment was ligated to the KpnI site of the multiple cloning sites of pcDNA 3.1(-) or pFastBac plasmid (Invitrogen).

The site-directed mutagenesis of the epitope-tagged 5-HT_{1A} receptor with the substitution of the serines for the cysteines at the positions 417 and/or 420 was performed by the overlap extension PCR technique (Fig. 3.1) using the oligonucleotides containing mutation(s) corresponding to the above substitutions (Ponimaskin et al., 1998). First, two partial cDNAs were separately amplified from the wild type cDNA using the corresponding mutated sense (*Cys 417–Ser Forward*, *Cys 420–Ser Forward* and *Cys 417,420–Ser Forward*, respectively) or the antisense (*Cys 417–Ser Reverse*, *Cys 420–Ser Reverse* and *Cys 417,420–Ser Reverse*, respectively) primers and the sense (*HA-1A-Sense*) or the antisense (*1A-Antisense*) primers corresponding to the start and the end of the receptor cDNA. The two PCR products were purified from the agarose gel and used as templates for the second PCR. The second PCR reaction was performed using the *HA-1A-sense* and the *1A-antisense* primers. The PCR products were purified, cut by the KpnI endonuclease and cloned into the KpnI sites of the pFastBac or the pcDNA 3.1 vectors.

For the construction of the 5-HT_{1A} –YFP chimera (Fig. 3.2), the receptor cDNA was amplified with the primers *Chimera Sense-KpnI* and either *Chimera Antisense WT KpnI* for the wild type or *Chimera Antisense DM- KpnI* for the C417/420-S mutant receptors**.** The antisense oligonucleotides were designed to anneal at the 3’ end of the coding sequence to remove the translation termination signal TAA. The amplified fragments were purified, digested with the KpnI enzyme and ligated at the respective site of the pEYFP vector (Clontech), so that the YFP coding sequence was located in-frame at the 3’ end of the 5-HT_{1A} receptor. The nucleotide receptor sequences of the clones were verified by the Sanger DNA sequencing of the final plasmid.

Figure 3.1. Mutagenesis by the Overlap-Extension PCR. At the first PCR round, two partial fragments of the 5-HT1A receptor were amplified from the receptor cDNA with the point mutations introduced in the primer sequence. The partial cDNA copies produced in the first round were used as the template and at the same time as the primers for the synthesis of the full-length cDNA strains at the second PCR round. The full-length cDNA strains were then amplified with the side primers s1 and as2. S-sense, as-antisense, ORF-open reading frame (coding part of the sequence), *-the introduced point mutation, Kpn1-restriction site.



The PCR reactions were performed in 50 µl final volume. The standard PCR mixture contents were as follows: the PCR buffer (20 mM Tris/HCl, pH 8.4, 50 mM KCl), 2.5 mM $MgCl_2$, 10 pmol/µl of the sense and the antisense primers, 200 µM dATP, dCTP, dGTP, dTTP, 20 ng of the template DNA, 1 unit of the DNA polymerase (AmpliTaqGold from Applied Biosystems). The amplification was started with incubation for 5 minutes at 94°C followed by 30 amplification cycles (94°C 30 sec, 56°C 45 sec, 72°C 120 sec). In the second round of the overlap-extension PCR, 20-50 ng of the each of two fragments were used. The amplified fragments were purified by electrophoresis in the agarose gel.

Figure 3.2. Schematic representation of the YFP-tagged 5-HT_{1A} receptor. CMV promoter- cytomegalovirus promoter, YFP –yellow fluorescent protein, KpnI- restriction site, ORF- open reading frame.

3.2.2. Agarose Gel Electrophoresis

The DNA fragments were separated by agarose gel electrophoresis. The 1% agarose gels were prepared with TAE buffer. The DNA gels were stained with ethidium bromide (0.5 µg/ml in TAE) and the DNA bands were visualised under

ultraviolet light. The ethidium-bromide stained bands of DNA were excised from the gel and purified with the JetSorb DNA purification kit (Genomed).

3.2.3. Preparation of PCR products for cloning

The purified PCR product was treated with the restriction endonuclease KpnI to prepare the fragments with the "sticky" DNA ends for efficient cloning in the expression vector. The total volume of the reaction mixture was 50 μl. This mixture contained restriction buffer 1 (20 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.0), 100 μg/ml BSA, 1 mg of the purified PCR product and 0.5 units KpnI. The reaction was performed at 37 ^{o}C for 60 min. The DNA fragments were separated by agarose gel electrophoresis and purified by the JetSorb kit prior to the ligation.

3.2.4. Preparation of Expression Vectors for Cloning

The expression vectors (pFastbac, pcDNA3.1(-), pEYFP) were digested with the restriction endonuclease KpnI as described above, except that 5 μg of plasmid DNA and 2.5 units of KpnI were used in the reaction which was performed for 2 h. After digestion, the KpnI was inactivated by incubation at 65 ^{o}C for 20 min. The sticky ends of the digested expression vectors were dephosphorylated by incubation for 1 hour with 10 units of calf intestinal phosphatase to prevent self-ligation of the vector. The digested and dephosphorylated vectors were purified as described above.

3.2.5. Ligation

Ligation reactions were performed for 2 h at RT in 30 μl final volume and contained the ligation buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP 25 μg/ml BSA, pH 7.5), 20 ng of the digested vector, 20-100 ng of the digested

PCR product and 800 units of T4 DNA ligase. The ligation mixture was used directly for transformation of the DH5α or the *DH10Bac E.coli* competent cells.

3.2.6. Transformation of E.coli Competent Cells

The competent cells were defreesed on ice and placed into 12-ml polypropylene tubes. 30 μl of the ligation mixture were added to 100 μl of competent cells and mixed gently. After incubation for 30 minutes on ice, the cells were heat-shocked for 45 seconds at 37°C and placed on ice for 2 minutes, mixed with 900 μl of SOC medium and shaked in the water bath for 1h at 37 ^{o}C. The cells were collected by centrifugation and plated on the YT medium agar plates supplemented with appropriate selective antibiotics (50 μg/ml ampicillin for pcDNA3.1 and pFastBac vector, or 30 mg/ml kanamycin for the pEYFP vector). The plates were incubated overnight at 37°C.

3.2.7. Analysis of the Clones

The bacterial colonies were collected with the sterile pipette tip and grown in 2 ml of the YT medium with the respective antibiotics by overnight shaking at 37^{o}C. The plasmid DNA was isolated from the overnight culture by the alcaline lysis method. The bacterial cells were centrifugated (1000xg for 5 min) and resuspended in 0.2 ml of the RNAse –containing buffer P1. The cells were lysed by addition of an equal volume of the lysis buffer P2 and incubation for 5 min at RT. The genomic DNA was then precipitated by addition of 0.2 ml of the buffer P3 (pH 5.5). The precipitated genomic DNA was removed by the centrifugation for 10 min at 20000xg at 4^{o}C. The plasmid DNA was precipitated from the supernatant by addition of

isopropanol to 45% final concentration and centrifugation for 10 min at 20000xg, washed by 70 % ethanol, air-dried and dissolved in 50 μl of the TE buffer (pH 8.0).

Orientation of the inserts was analysed by digestion of the plasmid DNA with the restriction endonuclease PstI. The reactions were performed in 50 μl at 37^{o}C for 30 min. Each reaction mixture contained the restriction buffer 3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9), 500 ng of the DNA and 0.5 U of the PstI. The digestion products were analysed by agarose gel electrophoresis. The correctly oriented clones produced two fragments of 5342 and 847 bp for the clones in the pFastbac vector, 4856, 1435 and 625 bp for the colnes in the pcDNA 3.1(-) vector and 4484, 802 and 718 bp for the clones in the pEYFP vector.

3.2.8. Propagation and Purification of the Plasmid DNA

The clones with the correct insert orientation were selected for the plasmid amplification. For that, 100 μl of the overnight culture was inoculated in the 100 ml of the YT medium containing the respective antibiotic and incubated under shaking overnight at 37^{o}C. The plasmid DNA was isolated by the alkaline lysis method as described before, except for increase of the volumes of the P1, P2 and P3 buffers to 10 ml each. The precipitated genomic DNA was filtered off with a paper filter, and the plasmid DNA was purified from the solution using the Quiagen anion-exchange columns. The purification included the equilibration of the columns by flow-through of 10 ml QBT buffer and binding of the plasmid DNA with the silica matrix under low salt conditions. The binding was followed by washing of the impurities with 60 ml of the medium-salt buffer QC. The plasmid DNA was eluted with 15 ml of the high-salt buffer QF, precipitated by addition of isopropanol to the final concentration 45% and

sedimented by the centrifugation at 20000xg. for 30 min at 4^{o}C. The plasmid DNA precipitate was washed with 70% ethanol, air-dried and diluted in 1 ml of TE (pH 8.0). The procedure yielded about 1 μg of the pure plasmid DNA. The purified plasmid DNA was used for the sequence analysis, for the recombinant baculovirus construction or for the cell transfection.

3.3. Cell Culture and Transfection

3.3.1. Culturing of the Sf.9 Insect Cells

The Sf.9 cells (Smith et. Al. 1985) were grown in 75-mm^2 flasks in 10 ml of TC-100 medium supplemented with 10% of FCS and 1% of penicillin-streptomycin (PS) at 28°C. For sub-culturing, the medium was removed, the cells were scraped from the flask in 6 ml of the fresh TC-100 medium and resuspended. One sixth of the suspension was transferred into the new flask.

3.3.2. Construction of the Recombinant Baculovirus (Anderson et. al. 1996).

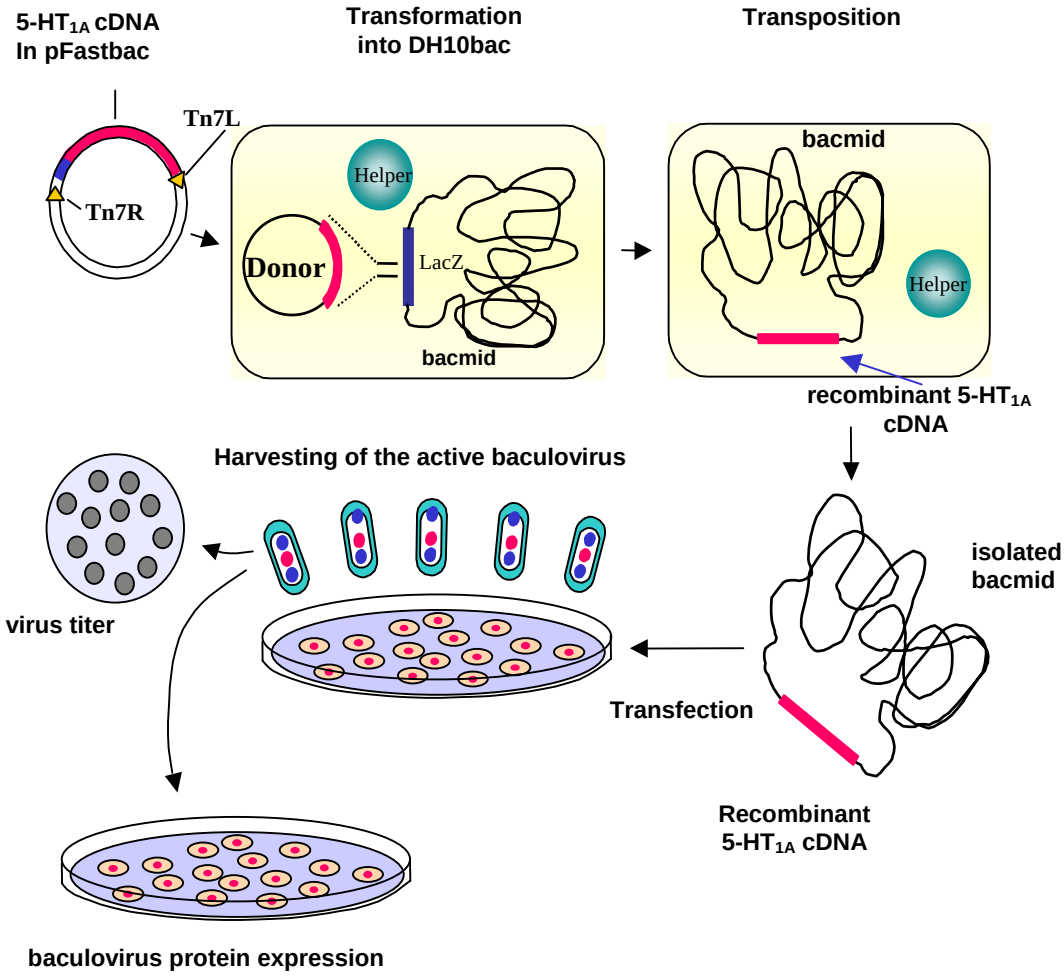
The baculovirus system allows to establish efficient overexpression of the functional mammalian proteins, and has been successfully used in many studies of the protein palmitoylation (Mouillac et al., 1992; Ponimaskin et al., 1998; Ponimaskin et al., 2002a; Ponimaskin et al., 2001) and some functional studies of the 5-HT$_{1A}$ receptor (Mulheron et al., 1994; Nebigil et al., 1995). For the construction of the recombinant baculoviruses encoding for the HA-tagged 5-HT$_{1A}$ wild type and the mutants, the *Escherichia coli* DH10Bac competent cells (Invitrogen) were transfected with the pFastbac plasmid DNA containing either the wild type or the mutated variants of the 5-HT$_{1A}$ receptor (Fig. 3.3). The DH10Bac cells contain bacmid and

helper DNA allowing for the site-specific recombination of the 5-HT_{1A} receptor cDNAs in the bacmid. The recombinant bacmid DNA was then purified by alkaline lysis and checked for the presence of the $5HT_{1A}$ receptor cDNA by PCR with the receptor-specific primers. The positive bacmid DNA preparations were transfected into the Sf.9 cells with the Cellfectin transfection reagent. For that, the cells were plated on the 35 mm dishes ($8*10^5$ cells/dish) one day before the transfection. The Cellfectin reagent was diluted 1 to 100 in the serum-free TC-100 medium. The 100 μl aliquot of the diluted Cellfectin was mixed with 200 ng of the purified bacmid DNA diluted in 100 μl of the serum-free TC-100 and incubated at room temperature for 30 min. After the incubation, 900 ml of the TC-100/10% FCS was added, and the DNA/Cellfectin mixture was transferred on the plate with the cells. After 4 hours the transfection mixture was replaced by 2 ml of the TC-100 medium with 10 % FCS and 1% P/S. One week after the transfection, the baculovirus–containing culture media was collected, purified of the cell debris by centrifugation (20 000 g for 10 min) and used for amplification of the recombinant baculovirus. The titres of the amplified baculoviruses were adjusted to $1x10^8$ pfu/ml.

3.3.3. Infection of the Sf.9 Cells with Baculovirus

The Sf.9 cells (10^6 cells per dish) were plated on the 35 mm dishes one day before the infection and incubated in a humid chamber at 29^oC. Next day the cells were infected with 10^5 pfu of the purified baculovirus. The cells were incubated with the virus for 60 min, being rocked every 15 minutes. After the incubation, the virus-containing medium was replaced by the TC-100/10% FCS/1% PS. The cells were subjected to the analysis 48 hours after the infection.

Figure 3.3. Construction of the Recombinant Baculovirus. The recombinant 5-HT_{1A} cDNA cloned into the pFastbac vector was transformed into the *DH10Bac* cells. Specific recombination between the vector and the bacmid DNA inside of the *DH10Bac* cells at the Tn7 sites led to the formation of bacmid DNA containing the 5-HT_{1A} receptor coding sequence. The bacmid DNA was purified and transfected into the Sf.9 cells, which allowed for the formation of active baculoviruses. The baculoviruses were harvested and amplified to the final titre of $1x10^8$ pfu/ml.



3.3.4. Culturing of the Mammalian Cells

The NIH-3T3 cells were cultured in the DMEM medium supplemented with glutaMAX II, 10% of FCS and 1% of PS on 10 cm plates. The CHO-K1 cells were cultured in the F-12 Ham medium supplemented with 10% of FCS and 1% of PS. For

a subculturing, the cells were treated with 3 ml of 0.05% trypsin (w/v)/0.02% EDTA (w/v), resuspended in 6 ml of the respective medium and 1/10 of the cell suspension was seeded on the new plate. All mammalian cells were grown at 37°C, 5% CO_2 and 95% humidity and passaged weekly (Phelan 1998).

3.3.5. Transient Transfection of the Mammalian Cells

The cells ($5x10^5$ cells per dish) were plated on the 35 mm dishes one day before the transfection. 6 µl of Lipofectamine 2000 were diluted in 200 ml of the OPTIMEM medium and incubated at RT for 5 minutes. 3 µg of the DNA were diluted in 200 µl of the OPTIMEM medium, mixed with the Lipofectamine 2000 solution and the mixture was incubated for 20 min at RT to form DNA/Lipofectamine 2000 complexes. After the incubation, 400 µl of the DNA/Lipofectamine 2000 mixture was mixed with 1600 µl of the culture medium containing 10% of FCS and placed on the plates with the cells for 4 hours. Then the transfection mixture was replaced by the culture medium with 10% of the FCS and 1% of the PS. The procedures were performed according to the manufacturers protocol (Pichet and Ciccarone, 1999).

3.3.6. Stable Transfection of the NIH-3T3 Cells

The NIH-3T3 cells were transfected using Lipofectamine 2000. One day after the transfection, the cells were trypsinised and 1/40 part of the cell suspension was plated on 10 cm dishes. 24 hours after the replating, the selective antibiotic Genetizin was added to the culturing media (DMEM/10 %FCS/1% PS) at the concentration of 1 mg/ml. The concentration of Genetizin was adjusted by the dose-response analysis. The cells were grown in the selective medium for two weeks. During the incubation, the medium was exchanged every 3 days. Single colonies were collected by the sterile

pipette tip, treated with 100 µl of the Trypsin-EDTA, resuspended and plated on separate 60 mm dishes. The stably transfected cell lines were tested for the expression of the recombinant protein by the Western blot. The amount of the receptor surface expression was analysed for the selected positive lines by specific radioligandligand binding.

3.4. Protein analysis

3.4.1. Metabolic Labeling and Immunoprecipitation

The Sf.9 cells ($1,5x10^6$ cells per dish) were grown in the 3.5 cm dishes were infected with the recombinant baculovirus encoding for the HA-tagged 5-HT_{1A} receptor. After 48 h, the Sf.9 cells were labeled with [^{35}S]-methionine (50 µCi/ml, >1000) or [^{3}H]-palmitic acid (300 µCi/ml, 30-60 Ci/mmol) for the time periods indicated in the figure legends. In some experiments, 5-HT or 8-OH-DPAT were added to the final concentrations as indicated in the figure legends. For some experiments, cycloheximide (50 µg/ml) was added 10 min prior to the incubation with [^{3}H]-palmitate or [^{35}S]-methionine to block protein synthesis. For the pulse-chase experiments the cells were incubated after the labeling with complete TC-100 medium supplemented with 100 µM unlabeled palmitate and 50 µM sodium pyruvate. After the labeling, the cells were washed once with the ice-cold PBS and lysed in 600 µl of the NTEP buffer (0.5% NP-40, 150 mM NaCl, 50 mM Tris/HCl (pH 7.9), 5 mM EDTA, 10 mM Iodoacetamide, 1 mM PMSF) for 15 min on ice. The insoluble material was pelleted (5 min, 20.000 x *g*) and the anti-HA antibodies (Santa Cruz) were added to the resulting supernatant at the dilution 1:60. After overnight agitation

at 4° C, 30 µl of Protein A-Sepharose CL-4B was added, and the samples were incubated under gentle rocking for 2 h. After brief centrifugation, the pellet was washed twice with the ice-cold NTEP-buffer and the immunocomplexes were released from the beads by incubation for 30 min at 37°C in the non-reducing electrophoresis sample buffer. The radiolabeled polypeptides were analysed by the SDS-PAGE on 12% acrylamide gels under the non-reducing conditions and visualized by fluorography using Kodak X-Omat AR films. Densitometric analyses of the fluorograms were performed by Gel-Pro Analyser Version 3.1 Software. The protocols are modified from Current Protocols in Cell Biology **(**Bonifacino, 2000**)**

3.4.2. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

10% polyacrilamide gel was used for the separation of proteins with the size 60-120 kDa, while 12% gel was used for the separation of proteins with the molecular mass below 60 kDa. The proteins in the sample buffer (62,5 mM Tris-HCl, pH 6.8, containing 20% glycerol, 6% SDS and 0,002% bromphenol blue) were loaded on the top of the gel and migrated under the influence of the applied electrical field from the cathode to the anode in the running buffer (25 mM Tris/HCl, 192 mM Glycin, 0.1% SDS, pH 8.4). To define the protein size, the molecular weight marker was loaded in parallel with the probes. The protocol is modified from Laemli (1970)

3.4.3. Western blot

After completion of the electrophoresis, the gel was removed, pre-equilibrated in the transfer buffer (25 mM Tris/HCl, 192 mM glycine, 20% (v/v) methanol, pH 8.3) and placed on top of the nitrocellulose membrane (0.2 µm pore diameter). Three sheets of Whatman filter paper pre-soaked in the transfer buffer were placed above

and below of the gel and the membrane. Transfer of proteins to the membrane was performed by application of the current (3 mA per 1cm^2) for 2 h.

After the transfer, the membranes were incubated in 5% dry milk solution in the PBS-Tween buffer for 30 min and incubated with the primary antibodies dissolved in the PBS-Tween overnight at 4^{o}C. The membranes were washed 3 times 10 minutes with 30 ml of the PBS-Tween, incubated with the secondary horseradish-peroxidase conjugated antibodies for 1 h at RT, washed again and subjected to the detection. All the incubations were performed in the PBS-Tween.

For protein detection, the chemiluminescent ECL Western Blotting Detection system (Amersham Biosciences) was used. The membranes were placed on a piece of SaranWrap film, covered by the detection reagent and incubated for 1 minute at RT. The membrane was covered with the second piece of the SaranWrap and an excess of the detection reagent was pressed out. The protein bands were visualised by exposure to the Kodak-X-OMAT AR films. The protocol is modified from Laemli (1970)

3.5. Hydroxylamine Treatment and Fatty Acid Analysis

The polyacrilamide gels containing the 5-HT$_{1A}$ receptor labeled with [^{3}H]-palmitic acid were fixed (10% acetic acid, 10% methanol) and treated overnight with 1 M hydroxylamine (pH 7.5) or 1 M Tris (pH 7.5) under gentle agitation. The gels were then washed in water and rocked for 30 min in dimethylsulfoxide (DMSO) to wash out the cleaved fatty acids and visualised by the by exposure to the Kodak-X-OMAT AR films.

For the fatty acid analysis, [^{3}H]-palmitate labeled 5-HT$_{1A}$ receptor was isolated by immunoprecipitation and SDS-PAGE. The band corresponding to the receptor

protein was excised and fatty acids were cleaved by the treatment of the dried gel slices with 6 N HCl for 16 h at 110° C. The fatty acids were then extracted with hexane and separated into the individual fatty acid species by thin layer chromatography using acetonitril/acetic acid (1:1, v/v) as a solvent. The radiolabeled fatty acids were visualized by fluorography. The protocols used were optimised previously (Ponimasin et. al. 2001)

3.6. Indirect Immunofluorescence

48 h after the infection, the Sf.9 cells grown on coverslips were fixed with paraformaldehyde (3% in PBS) for 15 min. The paraformaldehyde was quenched with 50 mM glycine for 15 min and the cells were washed three times with PBS. The cells were then permeabilized with 0.1% saponin for 5 min and incubated for 1 h with the anti-HA antibodies diluted 1:200 in PBS containing 2% BSA (PBS/BSA). The second antibodies (anti-mouse Alexa546-conjugated, diluted 1:1000 in the PBS/BSA) were applied for 1 h and the unbound antibodies were washed off at every step with PBS. Finally, the coverslips were mounted in 90% (v/v) glycerol in PBS. The protocol is modified from Donaldson (1998). The cells were monitored with the confocal laser-scan microscope LSM510 (Zeiss) using the Zeiss filter set 15 (BP 546/12, LP 590)

3.7. Assay for the [^{35}S] GTPγS Binding

The Sf.9 cells were infected with the respective baculovirus stocks as described above. Two days after the infection, the cells were scraped from the dishes, washed with 0.9% NaCl supplemented with 2 μg/ml aprotinin and 100 μM PMSF and resuspended in 2 ml of Hepes-EDTA containing 100 μM PMSF, 2 μg/ml aprotinin

and 10 μg/ml leupeptin. The cells were homogenised by 30 strokes of the Teflon pestle (1600 rpm) and the cell debris was removed by centrifugation at 100xg for 5 min. The supernatant was transferred into new tubes and centrifuged at 16000xg for 30 min at 4°C. The membrane pellet was washed and resuspended in the Hepes-EDTA buffer to adjust the protein concentration to 3 mg/ml. The samples were stored at –80°C.

The agonist-promoted binding of [^{35}S]guanosine 5´-(3-O-thio)triphosphate to the different G-proteins induced by a stimulation of the 5-HT_{1A} receptors was performed according to the method described previously (Ponimaskin et al., 1998). Briefly, 2 μl of the membranes from the Sf.9 cells expressing the 5-HT_{1A} receptor wild type or the acylation-deficient mutants and the G-protein α subunits (G_{i1}, G_{i2}, G_{i3}, G_s, G_{12}, G_{13}) together with the $G\beta_1\gamma_2$ subunits were resuspended in 55 μl of the GTPγS buffer A (50 mM Tris/HCl, 2 mM EDTA, 100 mM NaCl, 3 mM $MgCl_2$ and 1 μM GDP). After addition of [^{35}S] GTPγS (1300 Ci/mmol) to a final concentration of 30 nM, the samples were incubated for 5 min at 30°C in the presence or the absence of 1 μM 5-HT. The reaction was terminated by addition of 600 μl of the GTPγS buffer B (50 mM Tris/HCl, pH 7.5, containing 20 mM $MgCl_2$, 150 mM NaCl, 0,5% NP-40, 200 μg/ml aprotinin, 100 μM GDP and 100 μM GTP). The samples were incubated on ice for 30 minutes, mixed with 100 μl of 10% suspension of the protein A-sepharose and 10 μl of the antibodies directed against the appropriate Gα–subunits and agitated for 1 h at 4°C. The immunoprecipitates were washed three times with the GTPγS buffer B,

boiled in 0.5 ml of 0.5 % SDS and the radioactivity was measured by the scintillation counter.

3.8. Assay for the Receptor-Ligand Binding

3.8.1. Saturation Binding Experiments

The membranes from the Sf.9 cells expressing the wild type (WT) or the mutated 5-HT_{1A} receptors were dissolved in the buffer containing 20 mM Hepes (pH 8.0), 2 mM $MgCl_2$, 1 mM EDTA, 0.1 mM PMSF, 10 μg/ml leupeptin, and 2 μg/ml aprotinin. The binding assay with [^{3}H]5-HT was performed as described previously (Butkerait et al., 1995; Hall et al., 1985). Briefly, 100 μl of the ligand binding buffer containing 50 mM Tris (pH 7.7), 0.1 % ascorbic acid, 20 μM pargyline and 1 to 250 nm of [^{3}H]5-HT was added to the aliquot of the membrane fraction containing 20 μg of protein. The non-specific binding was determined by the addition of 100 μM of the unlabeled 5-HT. After 30 min incubation at 20^o C, the reaction mixture was loaded on 20 μm PVDV membranes (Corning, Germany) pre-soaked in 0.5 % polyetilenimine. The membranes were washed with the ice-cold binding buffer and the radioactivity was measured by the scintillation counter. The data were fitted with the one-site saturation binding model by the Pharmacology module of the Sigma Plot 8.02 software (SPSSInc., 2002).

3.8.2. Analysis of the Receptor Surface Expression

The NIH 3T3 or CHO cells transfected with the receptor cDNA were grown on 24-well plates, starved in the respective medium without FCS for 16 hours prior the assay and washed 2 times with ice-cold PBS. For the ligand binding, the cells were

incubated with 50 nM [^{3}H]5-HT or 30 nM [^{3}H] 8-OH DPAT diluted in the culture medium with 0.2 % BSA for 60 min on ice. The cells were washed 5 times with ice-cold PBS and lysed in 0.2 M NaOH. The bound radioactivity was measured by the scintillation counter. Non-specific binding was determined by the addition of 100 μM unlabeled 5-HT. The protocol is modified from Varrault et. al. (1992)

3.9. The cAMP Accumulation Assay

These experiments were done in collaboration with M. Sebben and A. Dumuis, UPR CRNS, Montpelier, France.

The NIH-3T3 cells were selected for the assay, as these cells do not contain specific binding sites for the 5-HT$_{1A}$ agonist 8-OH-DPAT (Varrault et.al, 1992). The 5-HT$_{1A}$ receptor wild type and the acylation-deficient mutant cDNAs were cloned in the pcDNA3(-) vector and transfected in the NIH-3T3 cells by electroporation. Cells were diluted in DMEM (10^6 cells/ml) containing 10% dialysed fetal bovine serum (dFBS) and plated into 12-well clusters. Six hours after the transfection, the cells were incubated overnight in DMEM without dFBS containing 2 μCi [^{3}H] adenine/ml to label the ATP pool. The cells were washed and then incubated in 1 ml of culture medium containing 0.75 mM IBMX, 50 μM forskolin plus the drugs indicated in the figure legends for 15 min at 37°C. The reaction was stopped by replacing of the medium with 1 ml of ice-cold 5% trichloracetic acid. The cAMP accumulation was measured as described previously (Dumuis et al., 1988b). The amount of the expressed 5-HT$_{1A}$ receptor was measured as described in section 3.8.2.

3.10. Erk 1/2 Phosphorylation Assay

The Erk phosporylation assay was performed in the CHO cells, as this response mediated by the 5-HT_{1A} receptor was first described in the mesenchymal cell line (Della Rocca et al., 1999). The CHO cells were grown in the F-12 Ham medium supplemented with 10% FCS and 1% PS. For the experiments, the cells ($0.5x10^6$) grown in the 3.5 mm dishes were transfected with the recombinant 5-HT_{1A} receptor using the Lipofectamine 2000 transfection agent. Twenty hours after transfection, cells were starved in the F-12 Ham medium with 2% BSA and 1% PS for 16 h. Cells were then stimulated for 5 minutes with 10 μM 8-OH-DPAT at 37^{o}C under 5% CO_2, washed with PBS and lysed in the SDS-PAGE loading buffer. The lysates were separated by SDS/PAGE and subjected to Western blot analysis. The membranes were probed either with the antibodies raised against the phosphorylated Erk 1/2 (phospho-p42/44; dilution 1:2000) or against the total Erk (p42/44; dilution 1:1000). To analyse the receptor expression, the membranes were probed with the antibodies raised against the HA-epitope (dilution 1:1000). The relative amount of the phosphorylated and the total Erk 1/2 was quantified by densitometric measurements using the GelPro Analyser version 3.1 software. The surface expression of the wild type and the mutant receptors was adjusted to 450-500 fmol/mg protein, as assessed by the [^{3}H] 5-HT binding. The unspecific binding was determined in the presence of 100-fold excess of the specific 5-HT_{1A} agonist 8-OH DPAT.

3.11 Separation of the Detergent-Resistant Membranes (DRM) by Gradient Centrifugation

The DRM separation was performed as described before (Harder et al., 1998). The stably transfected NIH-3T3 cells were plated on the 35 mm dishes 2 days prior to the assay. The cells were washed twice with ice-cold PBS, scraped and centrifugated at 1000xg, 4^{o}C. For a cholesterol depletion, the cells were washed 2 times with the culture medium without FCS and antibiotics and treated for 45 minutes with 15 mM methyl-β-cyclodextrin (MβCD) diluted in the medium without FCS and antibiotics. The cells were subsequently lysed on ice for 30 min in the TNE buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 10% sucrose, 1% Triton X-100, 1 mM PMSF, 10 μM leupeptin, 2 μg/ml aprotinin) at the protein concentration 1.2 mg/ml TNE. The lysates were mixed with the double volume of 60% Optiprep^{TM} gradient medium (60% iodoxianol suspension in H_2O, Nycomed Pharma) to give a final concentration of 40 % Optiprep. The lysate/Optiprep^{TM} mixtures (600 μl) were placed on the bottom of a 2.5 ml ultracentrifuge tube (Beckman) and overlayed with 350 μl steps of each 35%, 30%, 25%, 20% and 0% Optiprep^{TM} in TNE (Table 3.1). The gradients were centrifugated for 5 h at 50 000 rpm in the TLS-55 rotor of the Beckman ultracentrifuge TL-100.

Six fractions corresponding to the initial gradient steps were collected from the top of the gradient (Table 3.1). The proteins from the each fraction were precipitated by 10 % TCA. The sediments were washed 2 times with acetone, air-dried,

resuspended in the SDS-PAGE sample buffer and analysed by SDS-PAGE followed by Western blot.

Table 3.1. Characterisation of the Optiprep™ Gradient Fractions

Concentration of Optiprep™, (%)	**40**	**35**	**30**	**25**	**20**	**0**
No of fraction (bottom-top)	**1**	**2**	**3**	**4**	**5**	**6**
Density, (g/ml)	**1,256**	**1,230**	**1,204**	**1,178**	**1,151**	**0,041**

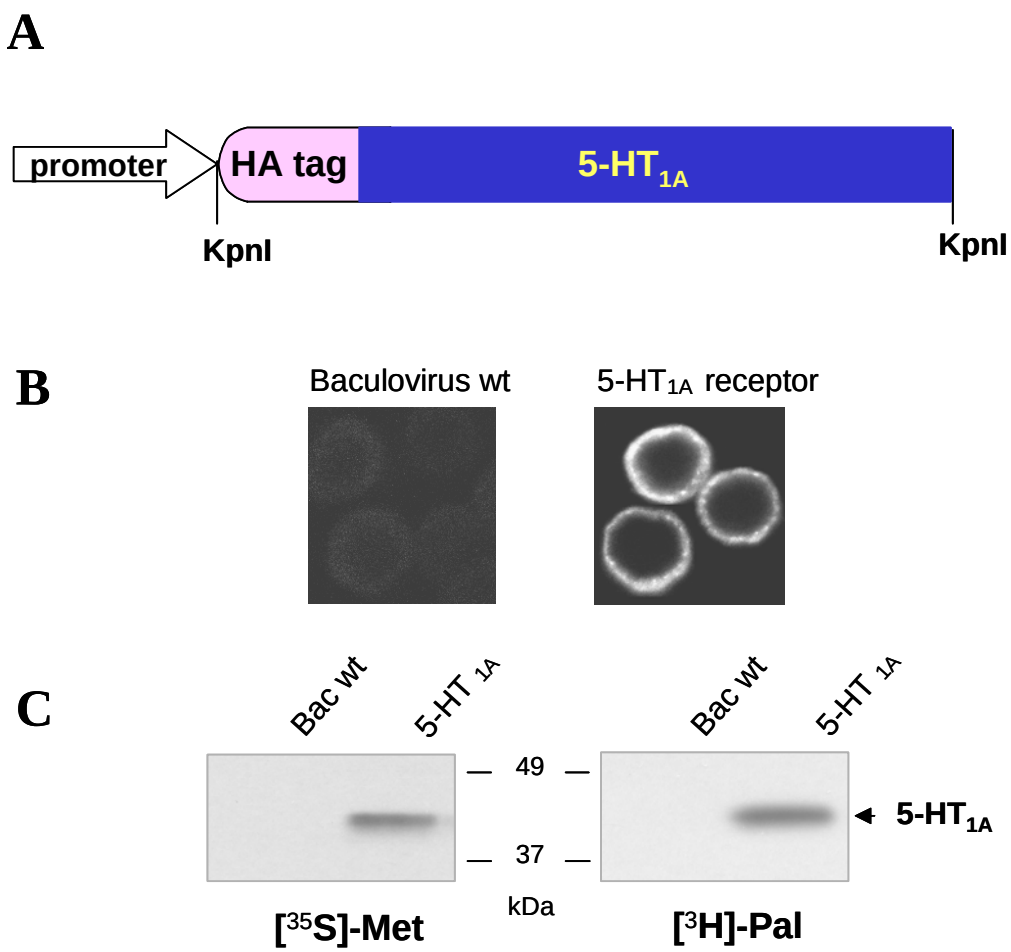
4. RESULTS

4.1 Expression and Palmitoylation of the 5-HT_{1A} Receptor

A high titre baculovirus stock containing the cDNA of the murine 5-HT_{1A} receptor tagged with a HA-epitope at the N-terminus (Fig. 4.1 A) was used for infection of Sf.9 insect cells. In order to monitor the expression and subcellular distribution of the receptor, infected Sf.9 cells were subjected for the immunofluorescence analysis (Fig. 4.1 B). The HA-tagged 5-HT_{1A} receptors were specifically detected by the anti-HA antibodies and localized mainly at the cell surface. The labeling with [^{35}S]-methionine followed by immunoprecipitation and SDS/PAGE revealed the single protein band with the molecular mass of approximately 46 kDa (Fig. 4.1 B, left panel). This corresponds to the predicted molecular mass of the 5-HT_{1A} receptor. The absence of specific bands in the immunoprecipitates from the non-infected or the baculovirus wild type infected Sf.9 cells confirmed that the 46 kDa band indeed represents the 5-HT_{1A} receptor.

To examine whether the 5-HT_{1A} receptor is acylated, the Sf.9 cells infected with recombinant baculovirus were metabolically labeled with [^{3}H]-palmitic acid. Such labeling, followed by immunoprecipitation and SDS-PAGE, revealed the single protein band of 46 kDa (Fig. 4.1 B, right panel) detectable only in the cells infected with the recombinant virus. This result demonstrates that the 5-HT_{1A} receptor efficiently incorporates [^{3}H]-palmitate.

Figure 4.1. Expression and Palmitoylation of the 5-HT$_{1A}$ Receptor in the Sf.9 Insect Cells. (A) Schematic representation of the cDNA encoding for the HA-tagged 5-HT$_{1A}$ receptor. **(B)** Sf.9 cells infected with a baculovirus encoding for the recombinant HA-tagged 5-HT$_{1A}$ receptor or with a baculovirus alone (Bac) were subjected to immunofluorescence staining with anti-HA antibodies. **(C)** Sf.9 cells expressing the 5-HT$_{1A}$ receptor were labeled for 2 h either with [^{35}S]-methionine (left panel) or with [^{3}H]-palmitate (right panel). Cell lysates were subjected to immunoprecipitation with the anti-HA antibodies followed by SDS/PAGE and fluorography. The exposure time was 1 day for [^{35}S]-methionine and 7 days for [^{3}H]-palmitate labeling. The molecular weight marker is indicated between the panels. Promoter- polyhedrin promoter.



Having shown that the 5-HT$_{1A}$ receptor is acylated, we analysed the chemical nature of the fatty-acid bond in order to distinguish between the amide-type and the ester-type fatty acid linkages. In contrast to the amide bond linkage, the S-ester and the

hydroxyester linkages are sensitive to the treatment with β-mercaptoethanol (McGlade et al., 1987). Moreover, the S-ester bond can be distinguished from the hydroxyester bond by its sensitivity to the treatment with neutral hydroxylamine (Kaufman et al., 1984). As shown in Fig. 4.2 A, the [^{3}H]-palmitate-derived radioactivity was sensitive to the treatment with increasing concentrations of the β-mercaptoethanol. Moreover, treatment of the [^{3}H]-palmitate-labeled 5-HT$_{1A}$ receptors with neutral hydroxylamine resulted in the cleavage of the label from the receptor (Fig. 4.2 B). These results demonstrate that the 5-HT$_{1A}$ receptor contains the thioester-linked acyl groups and no fatty acids linked through the amide- or the hydroxyester-bonds. To determine the identity of the receptor-bound lipids, the fatty acids were hydrolyzed from the gel-purified 5-HT$_{1A}$ receptor and separated by thin layer chromatography (TLC). Analysis of the TLC data revealed that the 5-HT$_{1A}$ receptor contains only the palmitic acid with no traces of the myristic or the stearic acids (Fig. 4.2 C).

4.2 The Palmitoylation of the 5-HT$_{1A}$ Receptor is a Stable Modification

To evaluate the role of the *de novo* protein synthesis in the receptor palmitoylation, the Sf.9 cells expressing the 5-HT$_{1A}$ receptor were incubated with [^{35}S]-methionine or [^{3}H]-palmitate in the absence or in the presence of the protein synthesis blocker cycloheximid. Surprisingly, this experiment revealed that the blockade of the protein synthesis results in the nearly complete inhibition of [^{3}H]-palmitate incorporation into the receptor (Fig. 4.3 A). Moreover, the inhibitory effect of the cycloheximid was not changed in the presence of the agonist.

Figure 4.2. The 5-HT$_{1A}$ Receptor is Modified with Palmitic Acid Attached via the Thioester-Type Bond. (A) The 5-HT$_{1A}$ receptor was labeled for 2 h with [^{3}H]-palmitate, immunoprecipitated and treated with increasing concentrations (5%, 10% or 15%) of β-mercaptoethanol for 30 min at 37 °C prior to SDS-PAGE and fluorography. **(B)** The Sf.9 insect cells expressing the 5-HT$_{1A}$ receptor were labeled with [^{3}H]-palmitate and subjected to immunoprecipitation and SDS-PAGE. The gel was treated with 1 M hydroxylamine (right panel) or 1 M Tris-HCl (left panel) prior to fluorography. The fluorogram shown is a representative of two independent experiments. **(C)** The 5-HT$_{1A}$ receptor labeled with [^{3}H] palmitate was immunoprecipitated and then subjected to the fatty acid analysis by thin layer chromatography (TLC). The fluorogram of the TLC plate was analysed with Gel-Pro Analyser software. The mobility of authentic [^{3}H]-palmitate, [^{3}H]-stearate and [^{3}H]-myristate standards are indicated by arrows.

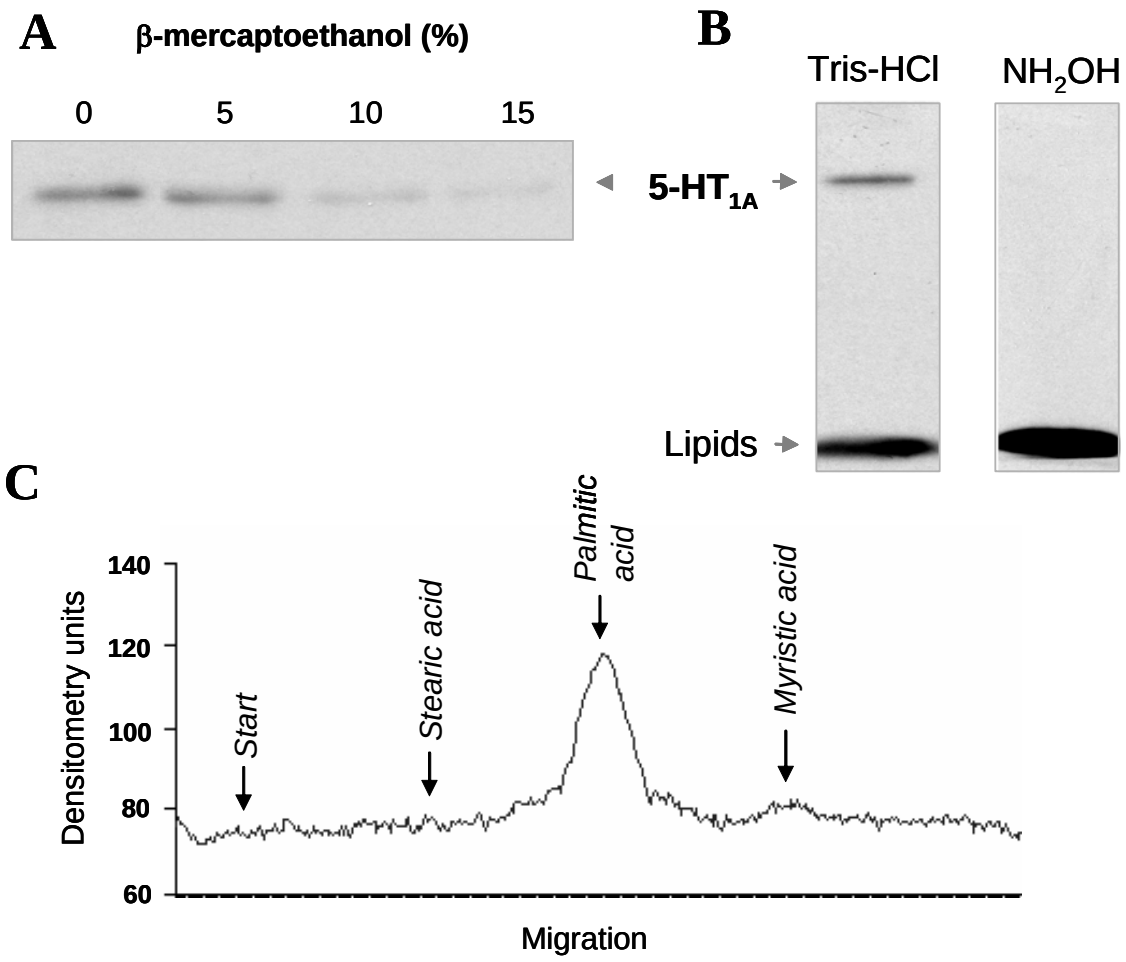
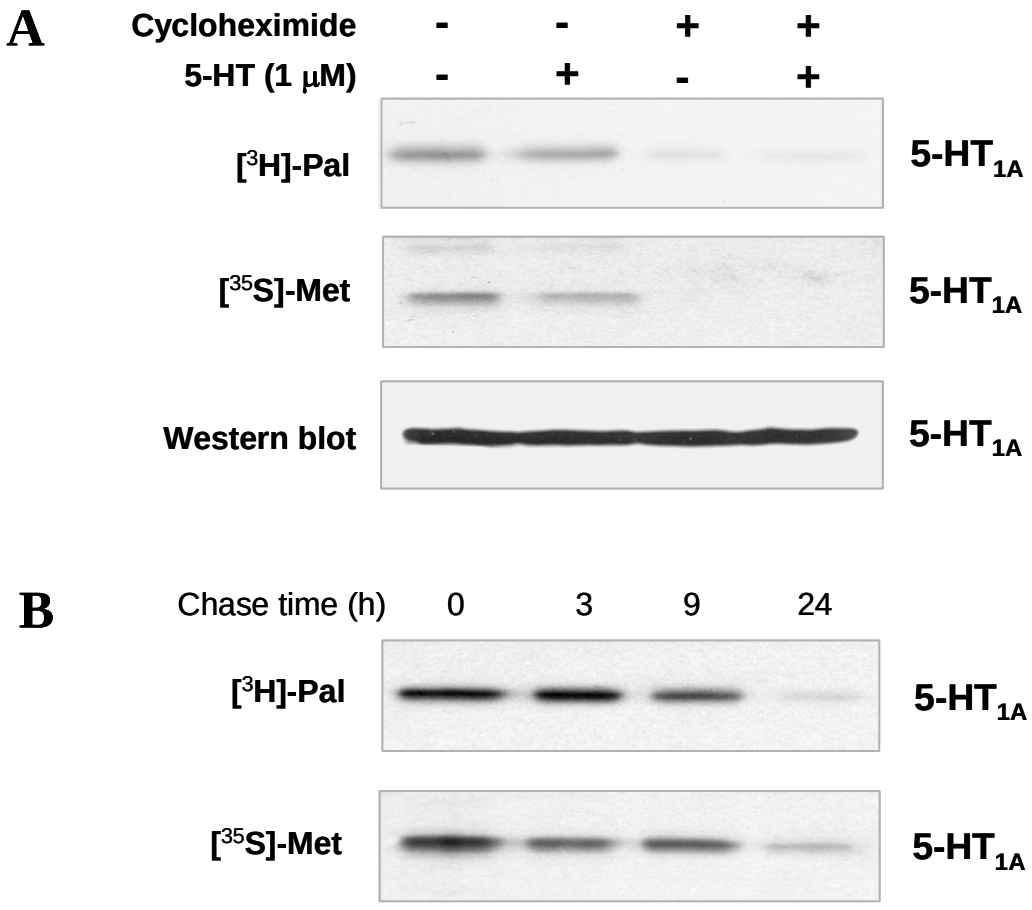


Figure 4.3. Palmitoylation of the 5-HT$_{1A}$ Receptor is a Stable Modification. (A) The Sf.9 cells expressing the 5-HT$_{1A}$ receptor were incubated for 60 min in the absence or the presence of the protein synthesis blocker cycloheximide (50 μg/ml) and either 1 μM 5-HT or vehicle (H_2O). In parallel with the cycloheximide treatment, the cells were labeled with [^{3}H]-palmitate or [^{35}S]-methionine. The cell lysates were then subjected either to immunoprecipitation, SDS/PAGE and fluorography or to Western blot to control the total amount of the 5-HT$_{1A}$ receptor. The fluorogram is representative of three independent experiments. **(B)** The Sf.9 cells were labeled with [^{3}H]-palmitate or [^{35}S]-methionine for 1 h and then chased with the medium containing the unlabeled palmitate or methionine for the periods indicated. During the chase time, the cycloheximide (50 μg/ml) was applied. The data demonstrate that palmitoylation of the 5-HT$_{1A}$ receptor is irreversible and occurs shortly after its synthesis.

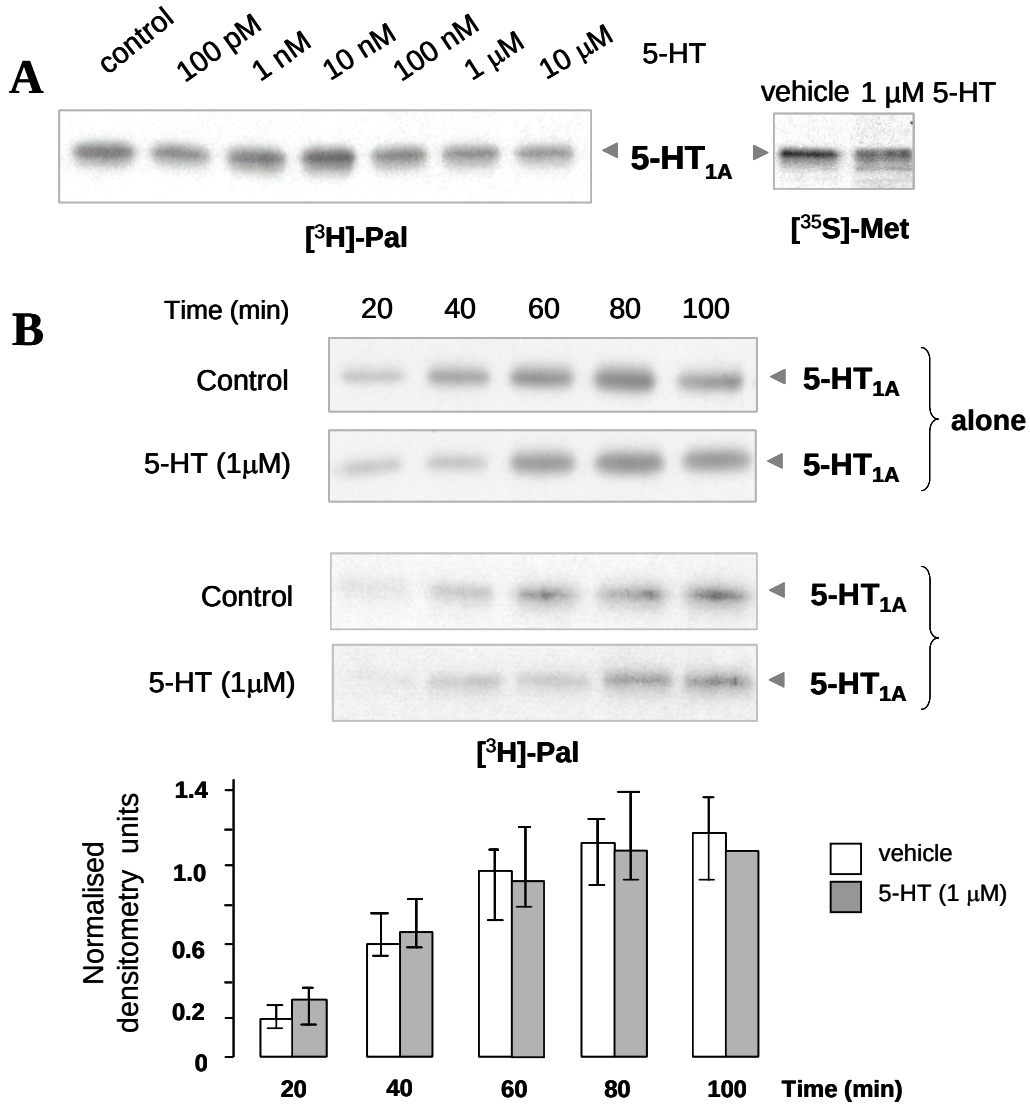


To further analyse the turnover rate of the 5-HT_{1A} receptor bound palmitate, we performed long-time pulse-chase experiments (Fig. 4.3 B). For that, purpose, the Sf.9 cells expressing the receptor were labeled with [^{3}H]-palmitate or [^{35}S]-methionine for 1 h (pulse), and the labeled cells were incubated in the medium without the labels, containing cycloheximide, for 3, 9 or 24 h (chase). As shown in the figure 4.3 B, the life-time of the [^{3}H]-palmitate labeling corresponds to the life-time of the receptor itself, demonstrating that no cleavage of the fatty acids from the receptor occurs during the chase period. Taken together, these data suggest that the palmitoylation of the 5-HT_{1A} receptor is a stable modification rather being limited to the pool of the newly synthesized receptor.

4.3. Activation of the 5-HT_{1A} Receptor Does Not Affect Receptor Palmitoylation

It has been previously demonstrated that the palmitoylation of the other member of the serotonin receptor family, the 5-$HT_{4(a)}$ receptor, is a dynamic process and that stimulation of the receptor by agonist increases the rate of the palmitate turnover (Ponimaskin et al., 2001). To test whether palmitoylation of the 5-HT_{1A} receptor may also be regulated by agonist, Sf.9 cells expressing the recombinant receptor were treated with increasing concentrations of 5-HT during the labeling of the cells with [^{3}H]-palmitate. The results shown in Fig. 4.4 A demonstrate that the stimulation of the 5-HT_{1A} receptor with the agonist does not result in any dose-dependent changes of the receptor palmitoylation. Labeling with [^{35}S]-methionine done in parallel demonstrated that the expression level of receptor was not affected upon the exposure to agonist (Fig. 4.4 A).

Figure 4.4. The Agonist Stimulation Does Not Affect the Palmitate Incorporation in the 5-HT$_{1A}$ Receptor. **(A)** The Sf.9 insect cells expressing the 5-HT$_{1A}$ receptor were incubated with [^{3}H]-palmitate or [^{35}S]-methionine for 60 min in the presence of the indicated concentrations of 5-HT. The receptor was immunoprecipitated, separated by SDS/PAGE and subjected to fluorography. **(B).** The Sf.9 cells expressing the 5-HT$_{1A}$ receptor alone or co-expressed with the Gi protein ($G\alpha_{i3}\beta_1\gamma_2$ subunits) were incubated with [^{3}H]-palmitate in the presence or the absence of 1 μM 5-HT for the indicated time periods. The incorporation of the radiolabel was detected by the immunoprecipitation followed by SDS/PAGE and fluorography. The fluorograms are representative of three independent experiments. Palmitate incorporation in the 5-HT$_{1A}$ receptor after 5-HT stimulation versus vehicle treatment (H_2O) during the time-course experiments is shown in **(C)** as the average (n=3). The range of values is shown by bars. The value obtained for the 100 min 5-HT stimulation was set to 1.



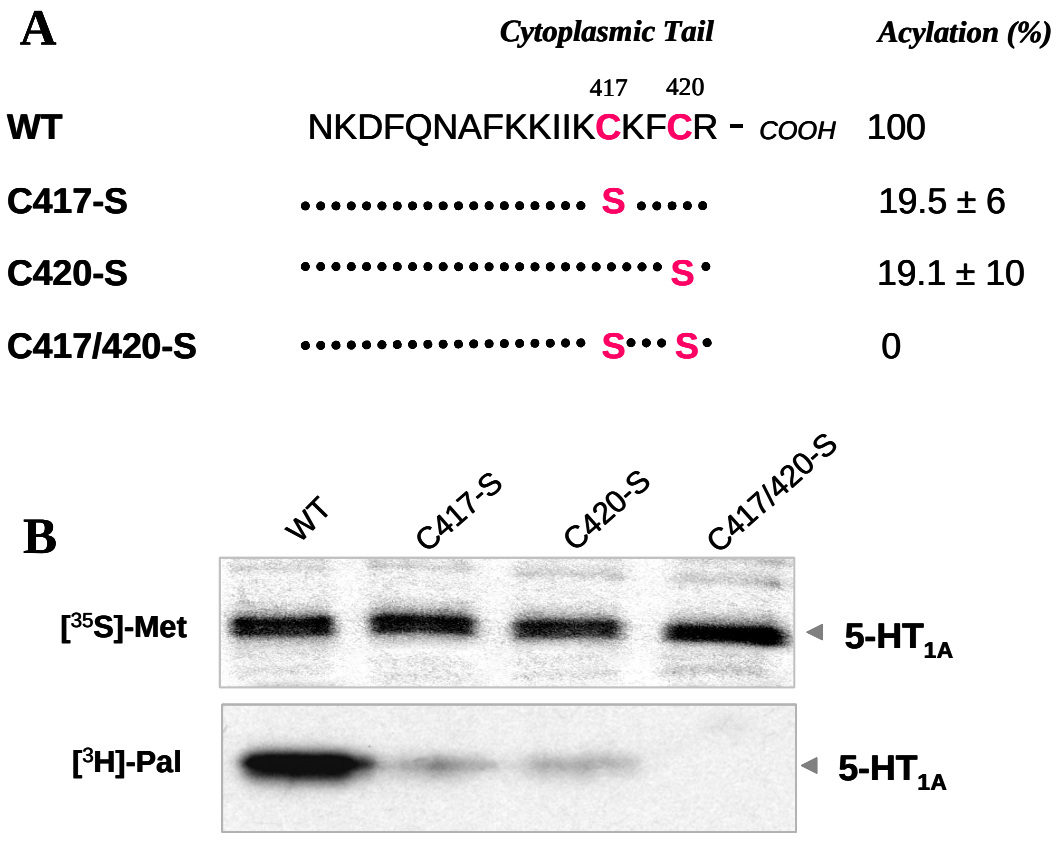
In order to obtain more detailed information on the dynamics of 5-HT_{1A} receptor palmitoylation, we studied the time-course of the agonist-induced incorporation of [^{3}H]-palmitic acid into the 5-HT_{1A} receptors. As shown in Fig. 4.4 B (control), the intensity of the radiolabel incorporation into the receptor increased steadily during the labeling period, reflecting the basal level of the palmitoylation. The kinetics of [^{3}H]-palmitate incorporation was then studied in the presence of the 5-HT. The results shown in the Fig. 4.4 B reveal that exposure to the agonist does not influence the efficiency of the radiolabel incorporation over the whole labeling period. The effect of agonist stimulation on the receptor palmitoylation was further analysed after the co-expression of the 5-HT_{1A} receptor with the heterotrimeric Gi-protein ($G\alpha i_3$-, $G\beta_1$- and $G\gamma_2$-subunits). Importantly, even in the coupled system, agonist stimulation has no effect on the efficiency of the receptor palmitoylation (Fig. 4.4 B).

4.4. Identification of the Palmitoylation Site(s)

From the analysis of a primary structure of the acylated GPCRs, it is known that the palmitoylation occurs exclusively on the cysteine residue(s) located in the cytoplasmic C-terminus of the receptors. To identify the potential palmitoylation site(s) within the 5-HT_{1A} receptor, we constructed a series of the mutant receptors in which the C-terminal cysteine residues 417 and/or 420 were substituted by serine residues (Fig. 4.5 A). All mutants were expressed in the Sf.9 insect cells by the baculovirus system and labeled with either [^{35}S]-methionine or [^{3}H]-palmitic acid followed by immunoprecipitation, SDS-PAGE and fluorography. Labeling with [^{35}S]-methionine revealed that the mutated

receptors were efficiently expressed along with the wild type (WT) receptor (Fig. 4.5 B, upper panel).

Figure 4.5. The 5-HT$_{1A}$ Receptor is Palmitoylated on Two C-terminal Cysteine Residues, Cys417 and Cys420. (A) The amino acid sequence of the C-terminal domain of 5-HT$_{1A}$ receptor wild type is shown with a single-letter code. The serine residues substituting for the corresponding cysteine residues are shown in red. The extent of palmitoylation for the substitution mutants was determined by densitometry and calculated as the ratio of the [^{3}H]-palmitate incorporation to the [^{35}S]-methionine signal. The value obtained for the wild type 5-HT$_{1A}$ receptor was set to 100 %. The data are presented as means ± S. E. (n=4). **(B)** The 5-HT$_{1A}$ receptor wild type and substitution mutants were expressed in the Sf.9 cells, labeled either with [^{3}H]-palmitate or [^{35}S]-methionine and subjected to immunoprecipitation, SDS/PAGE and the fluorography. The exposure time was 3 days for [^{35}S]-methionine and 42 days for [^{3}H]-palmitate labeling. A representative fluorogram is shown.



The single substitution of either the C-417 or the C-420 resulted in a significantly decreased, although not completely blocked palmitoylation. The double mutant in which both of the cysteine residues were replaced by serines did not reveal any detectable incorporation of [^{3}H]-palmitate even after the prolonged (6 weeks) exposure of the gel (Figure 4.5 B, lower panel). We quantified the level of palmitate incorporation for the individual mutants by densitometric analysis of the fluorograms after the [^{3}H]-palmitate labeling in relation to the expression level defined by the [^{35}S]-methionine incorporation. The analysis revealed that the incorporation of [^{3}H]-palmitate into the C417-S and the C420-S mutants was reduced to 19.5 ± 6% (n = 4) and 19.1 ± 9% (n = 4), respectively, in comparison with the wild type. Thus, we concluded that both of the cysteine residues C-417 and C-420 represent the palmitoylation sites on the 5-HT$_{1A}$ receptor.

4.5. Role of the Palmitoylation in the Coupling of the Receptor with G-proteins

To test for the functional significance of the 5-HT$_{1A}$ receptor palmitoylation, we analysed the interaction of the 5-HT$_{1A}$ receptor with the Gα subunits of the heterotrimeric G-proteins by using the GTPγS coupling assay (Ponimaskin et al., 1998). First, the Gα-subunits were co-expressed with the wild type receptor in Sf.9 cells (in all cases the appropriate Gα-subunit was co-expressed with the β_1 and the γ_2 subunits), and the agonist-promoted binding of [^{35}S] GTPγS to the Gα-subunit was assessed by counting radioactivity directly after immunoprecipitation with the antibodies directed against the appropriate Gα subunit (Fig. 4.6 A). When the wild type 5-HT$_{1A}$ receptor was co-expressed with Gα_{i1}, Gα_{i2} or Gα_{i3}, we measured a 1.7- to 2.5-fold increase in the [^{35}S]

GTPγS binding upon stimulation with 1 μM 5-HT. The result confirmed that the 5-HT_{1A} receptor effectively communicates with the G-proteins of the G_i family. In contrast, there was no coupling after co-expression of the receptor with the $G\alpha_s$, the $G\alpha_{12}$ or the $G\alpha_{13}$ subunits (Fig. 4.6 A).

We then tested an ability of the palmitoylation-deficient receptor mutants to couple with the $G\alpha_{i3}$ protein. In the case of the single mutants C417-S and C420-S, the agonist-dependent GTPγS binding was significantly decreased, when compared to the WT receptor. However, some significant activation of the $G\alpha_i$ protein over the basal level was still detectable (Figure 4.6 B). In contrast, when the non-palmitoylated receptor mutant C417/420-S was expressed, the relative activation of the $G\alpha_{i3}$ subunit after the agonist stimulation was completely abolished (Fig. 4.6 B). It is notable that the mutants as well as the WT 5-HT_{1A} receptor were expressed in a similar level, as assessed by the Western blot analysis (Fig. 4.6 B, inset). To exclude the possibility that this effect was mediated by the change in the ligand binding properties, we performed the pharmacological analysis of the pharmacological of the wild type and the mutant 5-HT_{1A} receptors expressed in the Sf.9 cells. We found that the pharmacological profile for the WT receptor was similar to that previously reported for the 5-HT_{1A} receptor expressed in the Sf.9 cells (Clawges et al., 1997). More importantly, the analysis of the palmitoylation-deficient mutants revealed that the replacement of the palmitoylated cysteines did not change their pharmacological properties (Fig. 4.7; Tab. 4.1). The binding affinity of [^{3}H] 5-HT for the wild type 5-HT_{1A} receptor was similar to that obtained for the mutants. Taken together, these data indicate a functional importance of the 5-HT_{1A} receptor palmitoylation for the coupling to the G_{i3}-protein.

Figure 4.6 Effect of Palmitoylation on the Coupling between the 5-HT$_{1A}$ Receptor and G$_i$-Protein. (**A**) Communication of the 5-HT$_{1A}$ receptor with different G-proteins. Membranes were prepared from the Sf.9 cells expressing the recombinant proteins as indicated and then incubated with [^{35}S] GTPγS in the presence of either H_2O (vehicle) or 5-HT (1 μM). The immunoprecipitations were performed with the appropriate antibodies directed against the indicated Gα-subunits. (**B**) The membranes were isolated from the Sf.9 cells co-expressing the recombinant Gα$_{i3}$- and the Gβ$_1$γ$_2$-subunits together with either the 5-HT$_{1A}$ receptor wild type or the acylation-deficient mutants. After the incubation with [^{35}S] GTPγS in the presence of H_2O (vehicle) or 1 μM 5-HT, the membranes were lysed and the Gα$_{i3}$-subunit was immunoprecipitated with the specific antibodies. The values obtained for the 5-HT$_{1A}$ receptor wild type after the agonist stimulation were set to 100%. The data points represent the means ± S.E. (n=6). A statistically significant difference between values is noted (*, $p < 0.01$). (***Inset***) Expression analysis for the WT and the acylation-deficient mutants. Samples from the parallel infections were used for the Western blot analysis with the Gα$_{i3}$- or the HA-specific antibodies.

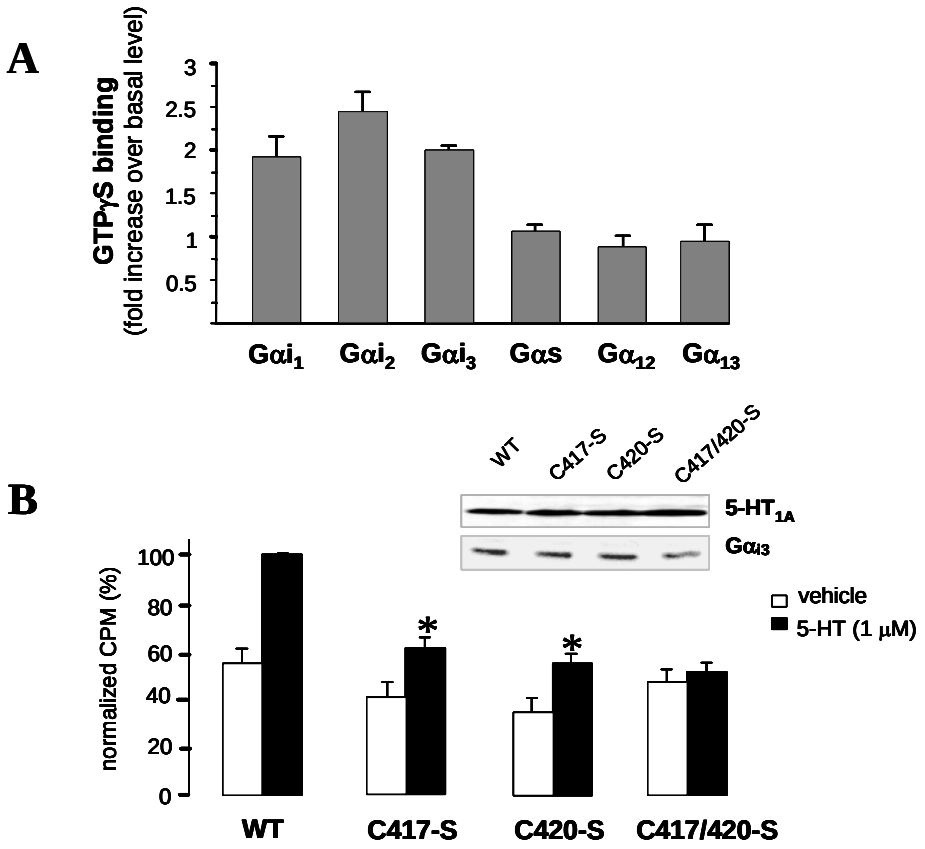


Figure 4.7. Mutation of the Palmitoylation Sites Does Not Change the Ligand-Binding Properties of the 5-HT$_{1A}$ Receptor. Saturation binding of [^{3}H] 5-HT with the WT and the palmitoylation-deficient 5-HT$_{1A}$ receptors was performed on the membranes prepared from infected Sf.9 cells. The non-specific binding did not exceed 5% of the specific binding and was subtracted from the total counts. Finally, the data were fitted to the one-site saturation model. Data points represent the means ± S.E. from at least three independent experiments performed in triplicate (n=9).

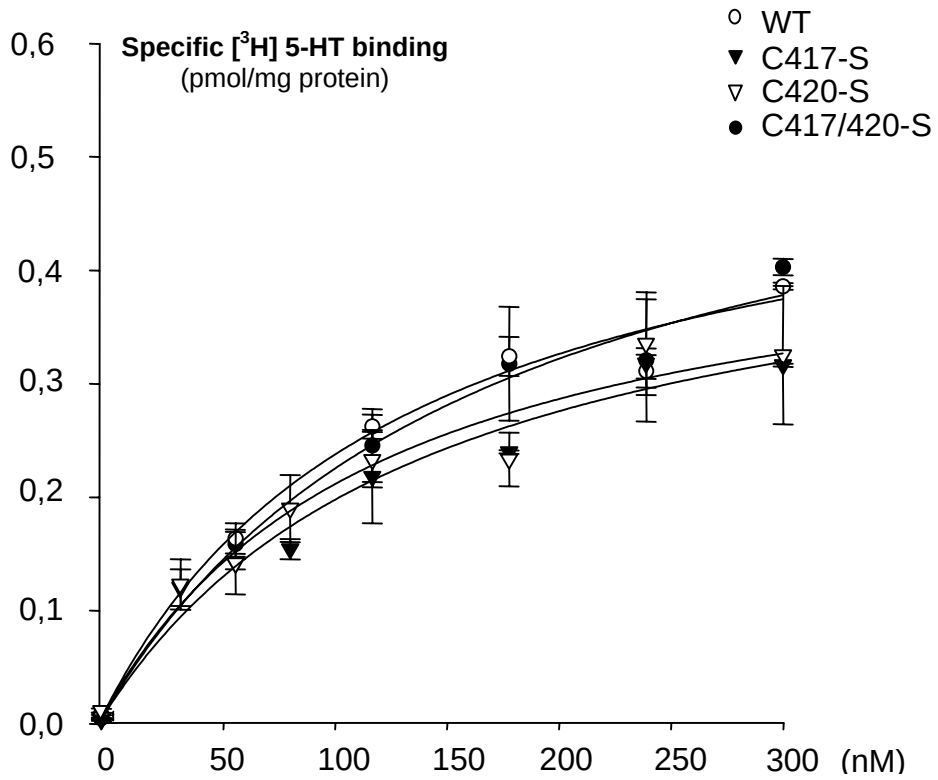


Table 4.1. Pharmacological Properties of the Palmitoylation-Deficient 5-HT$_{1A}$ Receptor Mutants Determined by the Saturation Binding of [^{3}H] 5-HT to the Membranes of the Sf.9 Cells. The Kd and the Bmax values were calculated by the one-site saturation fit. Data are expressed in the means ± S. E. (n=9)

	WT	**C417-S**	**C420-S**	**C417/420-S**
Kd (nM)	140 ± 66	101 ± 44	110 ± 23	109 ± 23
Bmax	0.590 ± 0.128	0.539 ± 0.044	0.473 ± 0.54	0.459 ± 0.082

(pmol/mg protein)				

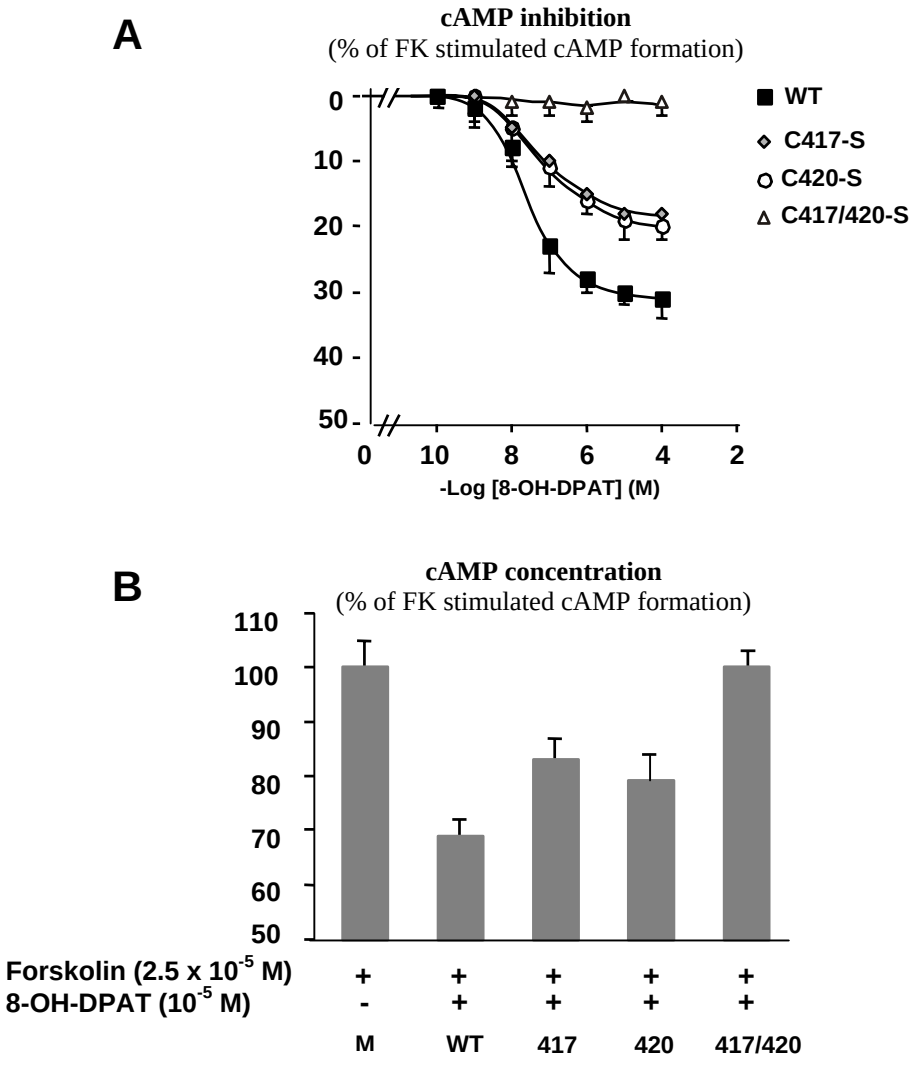
4.6. Role of the Palmitoylation in the 5-HT_{1A} Receptor Mediated Inhibition of the cAMP Formation

These experiments were done in collaboration with A. Dumuis and M. Sebben, UPR CRNS, Montpellier, France.

The functional role of the 5-HT_{1A} receptor palmitoylation for the inhibition a cAMP formation was then tested in the mammalian cell system (Fig. 4.8). Here the ability of the wild type and the mutant receptors to inhibit the forskolin-stimulated cAMP accumulation was analysed upon application of the specific 5-HT_{1A} receptor agonist 8-OH-DPAT (Hamon et al., 1984). The NIH-3T3 cells that do not contain any detectable [^{3}H]8-OH-DPAT binding sites (Varrault et al., 1992) were transfected with the pcDNA3.1(-) plasmid containing cDNA encoding for wild type and the acylation-deficient mutants of the 5-HT_{1A} receptor. The total expression level for the WT and the mutants was adjusted to 1500-1650 fmol/mg protein, which allowed for a quantitative analysis of the results.

Expression of the WT 5-HT_{1A} receptor resulted in the significant inhibition of the forskolin-promoted cAMP formation upon the receptor stimulation with the 8-OH-DPAT in a dose-dependent manner (Fig. 4.8 A). Replacement of any of the two palmitoylation sites was accompanied by a significant decrease in the capacity of the mutated receptors to inhibit the forskolin-stimulated cAMP formation. While the maximal inhibition of the cAMP formation obtained for the WT 5-HT_{1A} receptor was 32 ± 3.6 %, for the palmitoylation-deficient mutants C417-S and C420-S this value was reduced to 17 ± 2.4% and 22 ± 4%, respectively (Fig. 4.8 B). In the case of the non-acylated 5-HT_{1A}

Figure 4.8. Effect of the 5-HT_{1A} Receptor Palmitoylation on the Inhibition of the Forskolin-Stimulated Adenylate Cyclase Activity was evaluated in the NIH-3T3 cells transiently expressing the 5-HT_{1A} receptor wild type, the C417-S, the C420-S and the C417/420-S mutants. **(A).** The intracellular cAMP level was measured after the treatment of the cells with forskolin at the increasing concentrations of 8-OH-DPAT. The data points represent means ± S.E. (n=12) **(B).** The effect of the saturating concentration (10^{-5} M) of 8-OH-DPAT. Levels of the cAMP accumulation were measured after 15-min incubation and expressed as a percentage of the cAMP accumulation in mock-transfected cells. The percentage conversion of [^{3}H] ATP to [^{3}H] cAMP in the mock transfected NIH-3T3 cells was 0.115 ± 0.013. Each value is the mean ± S.E. of at least four independent experiments, performed in triplicate. In all cases the level of the surface expression for the receptors was adjusted to 1500–1650 fmol/mg protein. M-mock transfected cells, WT - wild type, 417 - C417-S, 420 - C420-S, 417/420 - C417/420-S.



receptor mutant, the inhibitory potential of the receptor was completely abolished and exposure to agonists had no effect on the intracellular cAMP level (Fig. 4.8 B). Analysis of the dose-dependent inhibition of cAMP formation upon application of the 8-OH-DPAT revealed that the EC_{50} value for the single mutants was approximately 2.5 times higher than that obtained for the WT 5-HT_{1A} receptor. We calculated an EC_{50} of 127 ± 4 nM for the Cys417-Ser, 140 ± 7 nM for the Cys420-Ser mutants and 52 ± 6 nM for the WT. These data confirmed the results obtained for $G\alpha_{i3}$ coupling in the Sf.9 insect cells and point to a functional significance of the 5-HT_{1A} receptor palmitoylation in the $G\alpha_i$-mediated signaling.

4.7. Activation of the Erk 1/2 by the 5-HT_{1A} Wild type Receptor and the Acylation Deficient Mutants

In addition to the $G\alpha_i$-mediated inhibition of the adenylate cyclase, the 5-HT_{1A} receptor modulates the activity of the extracellular signal-regulated kinase (Erk) via a $G\beta\gamma$-mediated pathway (Garnovskaya et al., 1996). The Erk is switched to the activated form by its phosphorylation on the threonine 202 and the tyrosine 204 residues. Therefore, we analysed the ability of the 5-HT_{1A} receptor WT and the palmitoylation-deficient mutants to activate the Erk by the Western blot analysis with the antibodies directed against the phosphorylated form of the Erk 1/2. In parallel, the expression level of the Erk 1/2 as well as of the 5-HT_{1A} receptor was verified by a Western blot with antibodies against the total Erk or against the HA-epitope, respectively. The surface expression level for the

WT and the mutant receptors was adjusted to 450-500 fmol/mg protein, which allowed for the quantitative analysis of the results.

Figure 4.9. Activation of the Erk 1/2 kinase by the 5-HT$_{1A}$ Receptor Wild Type and Acylation-Deficient Mutants. (A) The CHO cells were transiently transfected with the 5-HT$_{1A}$ receptor WT or mutants. In all cases the receptor surface expression was adjusted to 450-500 fmol/mg protein as assessed by the ligand binding. The transfected cells were treated with 10 µM 8-OH-DPAT or H_2O (vehicle) for 5 min. The proteins were analysed by SDS/PAGE and Western blot. The membranes were probed either with the antibodies raised against the total (phosphorylated and non-phosphorylated) Erk (upper panel) or against the phosphorylated Erk (lower panel). To analyse the 5-HT$_{1A}$ receptor expression, the membranes were probed with the antibodies raised against the HA-epitope (right panel). Fluorograms are representative of four independent experiments. **(B)** Quantification of the Erk phosphorylation for the receptor WT and the substitution mutants was performed by densitometry and calculated as the ratio of the Erk phosphorylation signal normalized to the total Erk expression. Each value represents the mean ± S.E. (n = 4). The statistically significant differences between the values are noted (*, $p<0.05$; **, $p<0.01$)

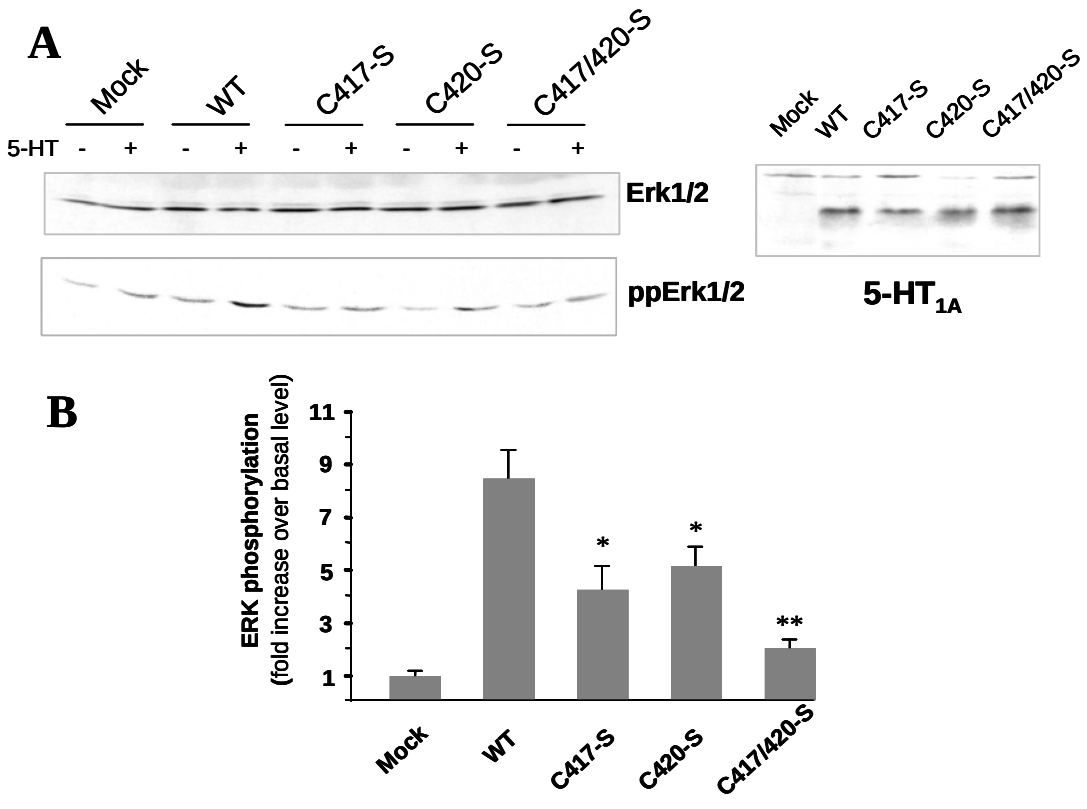


Figure 4.9 demonstrates that the agonist treatment of the CHO cells transiently transfected with the WT 5-HT_{1A} receptor resulted in the approximately 8-fold increase of the phosphorylation and thus of the activation of the Erk 1/2. For the single acylation mutants C417-S and C420-S we obtained a significantly reduced increase (approximately 4-fold) of the agonist-induced Erk 1/2 phosphorylation. In the case of the non-acylated mutant C417/420-S, treatment with the agonist induced only a very weak increase (approximately 1.8-fold) in the phosphorylation of the Erk. These data suggest the importance of the 5-HT_{1A} receptor palmitoylation for the signaling through the Gβγ-mediated pathway, in addition to the $G\alpha_i$-mediated signaling.

4.8. Possible Role of the Palmitoylation for the Targeting of the 5-HT_{1A} Receptor to the Detergent-Resistant Membrane Subdomains

Palmitoylation has been shown to be involved in the recruitment of several proteins to the membrane subdomains, like lipid rafts or caveolae. Therefore, we compared the distribution of the wild type and the palmitoylation–deficient mutant (C417/420-S) of the 5-HT_{1A} receptor in the detergent-resistant membrane subdomains (DRMs). For that, NIH-3T3 cells stably expressing for either the wild type or the mutant receptor were solubilised in the cold Triton-X100 and the lysates were subjected to the ultracentrifugation in the Optiprep^{TM} density gradient, as described in “Experimental procedures”. The immunoblot analyses of the gradient fractions prepared from the cells stably expressing the wild type 5-HT_{1A}-YFP (surface expression of 304±62 fmol/mg protein) (Fig. 4.10 A) demonstrated that the portion of the 5-HT_{1A} receptor was located in

the low density membrane fractions 3 and 4 (30 % and 25 % Optiprep^{TM}, respectively). The DRM-located buoyant part of the $G\alpha_{i3}$ protein and the caveolae marker Caveolin 1 were also located in these fractions. The major fraction of the wild type receptor was, however, localized in the high density fractions 1 and 2 (40 % and 35 % Optiprep^{TM}) (Fig. 4.10 A). The distribution of a non-DRM marker transferrin receptor was limited to the high density fractions 1 and 2. Interestingly, the palmitoylation-deficient mutant C417/420-S stably expressed in the NIH-3T3 cells (surface expression of 635±64 fmol/mg protein) was apparently depleted from the low density fractions 3-4, while distribution of the $G\alpha_{i3}$ protein, the caveolin 1 and the transferrin receptor was not affected (Fig. 4.10 A). Quantitative analysis of the fluorograms revealed that approximately 30 % of the wild type receptor are localized in the low density fractions 3 and 4, while in the case of C417/420-S mutant only the fraction of approximately 4% was found in the low density fractions (Fig. 4.10 B). Similar distribution was found when the HA-tagged wild type and the acylation-deficient C417/420-S mutant 5-HT_{1A} receptors were transiently expressed in the CHO cells (data not shown).

It has been shown that an integrity of the DRMs can be disturbed by the specific cholesterol depletion (Harder et. al. 1998). Thus, amount of the DRM-associated proteins in the low density fractions can be reduced by the treatment of the cells with the cholesterol-depleting agent methyl-β-cyclodextrin prior to the lysis. This can serve as an additional specific test for the localization of the proteins in the buoyant cholesterol-enriched membrane subdomains. Localization of the wild type 5-HT_{1A} receptor and the $G\alpha_i$ protein in the low density gradient fraction was sensitive to the cholesterol depletion. As shown in the Fig. 4.11, when the NIH-3T3 cells stably expressing the wild type 5-

HT_{1A} receptor were treated with the methyl-β-cyclodextrin prior to the Triton-X100 lysis, the amount of both the receptor and the $G\alpha_i$ protein in the low density fractions 3 and 4 was decreased, while the distribution of the non-DRM marker transferrin receptor did not change (Fig. 4.11). Altogether, these data suggest that the palmitoylation of the 5-HT_{1A} receptor may represent the signal for the receptor targeting to the cholesterol enriched DRMs.

Figure 4.10. Mutation of the Palmitoylation Sites Affects Location of the 5-HT$_{1A}$ Receptor in the Detergent-Resistant Membranes. The NIH-3T3 cells stably expressing either the YFP-tagged wild type 5-HT$_{1A}$ receptor or the Cys417/420-Ser mutant, were lysed with the cold 1% Tryton-X100 and the lysates were ultracentrifugated in the Optiprep^{TM} density gradient. The gradient fractions were analysed by SDS-PAGE and Western Blot. **(A)** The wild type 5-HT$_{1A}$-YFP (about 80 kDa) is enriched in the low density fractions 3 and 4 together with the Gα_i protein and the Caveolin 1, while the mutant C417/420-S receptor is almost excluded from these fractions. The transferrin receptor was used as the non-DRM marker. Numbers of the gradient fractions (see Tab. 3.11 in „Experimental procedures“) are shown on the top of the fluorograms. The bottom and the top of the gradients are indicated below. The low density DRM fractions are highlighted by the red bar. **(B).** Relative amount of the receptor in the high density fractions 1+2 and the buoyant low density fractions 3+4. The quantitative analysis of the receptor distribution was performed by densitometry and calculated in percentage of the total of the all fractions. Data shown in means (n=3). The data range is shown by bars.

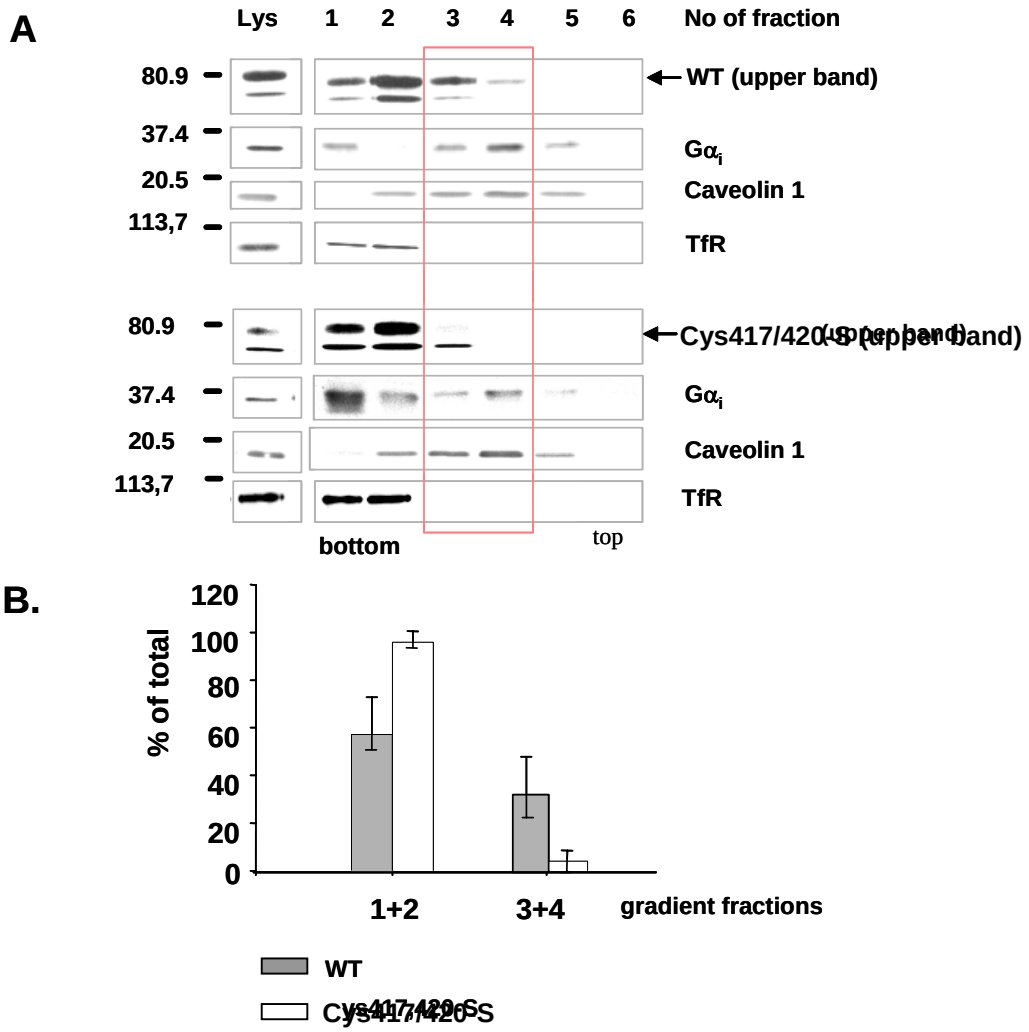
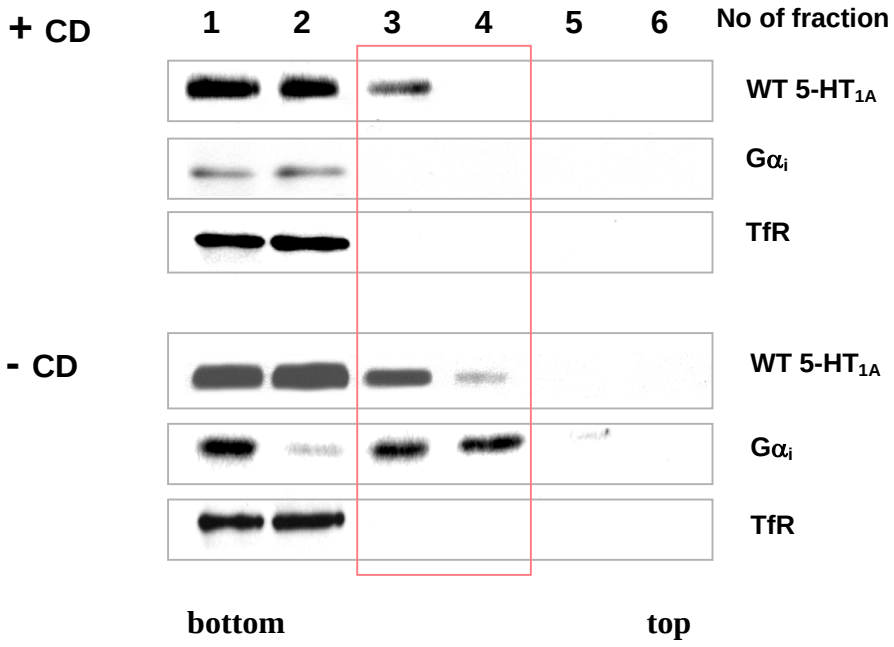


Figure 4.11. Cholesterol Depletion Reduces Localization of the Wild Type 5-HT$_{1A}$ Receptor and the Gα_i Protein in the Detergent-Resistant Membranes. The NIH-3T3 cells stably expressing the YFP-tagged wild type 5-HT$_{1A}$ receptor were treated with 15 mM MβCD for 45 minutes (+CD) or left untreated (-CD), lysed with cold 1% Tryton-X100 and the lysates were subjected to the ultracentrifugation in the Optiprep^{TM} density gradient. The gradient fractions were analysed by SDS-PAGE and Western Blot. Representative fluorogram is shown (n=3). Numbers of the gradient fractions (see Tab. 3.11 in „Experimental procedures“) are shown on top of the fluorograms. The bottom and the top of the gradients are indicated below. The low density fractions, containing the DRM, are highlighted by the red bar.



5. DISCUSSION

5.1 Expression and Palmitoylation of the 5-HT_{1A} Receptor

The covalent attachment of the palmitic acid to proteins (acylation) is often a reversible modification, and the dynamic acylation has been demonstrated for a number of signaling proteins, including the G-protein coupled receptors (GPCR). In the present work, we demonstrate the palmitoylation of the 5-HT_{1A} serotonin receptor using the recombinant baculovirus expression in insect cells (Fig. 4.1 and 4.2A). The sensitivity of this modification to the nucleofilic and to the reducing agents (Fig. 4.2 B and C) indicated that the palmitic acid is attached to the receptor via a thioester linkage, presumably to the free sulfhydril group on cystein residue(s).

Treatment of the cells with the inhibitor of protein synthesis cycloheximide led to the diminished incorporation of [^{3}H]-palmitate into the receptor, indicating that there is no significant turn-over of the receptor-bound palmitate (Fig. 4.3 A). Furthermore, the results of the long-time pulse-chase experiments indicated that the fatty acid was stably attached to the receptor (Fig. 4.3 B), suggesting that the palmitoylation of the 5-HT_{1A} receptor is irreversible. Such stable palmitoylation is still unusual for the signaling proteins, in particular for the GPCRs that generally undergo repeated cycles of palmitoylation and depalmitoylation. The observed low rate of the palmitate exchange suggests that the depalmitoylation reaction is blocked or extremely slow. The resistance of the receptor-bound palmitate to the cleavage by thioesterases may be explained by the inaccessibility of the 5-HT_{1A} receptor palmitoylation site(s) to the depalmitoylating enzyme(s). This may be mediated by the orientation of the palmitate group within the membrane as well as by

the composition of neighbouring amino acids (see below). Alternatively, the very short (18 aa) C-terminus of the 5-HT_{1A} receptor could lack the specific motif(s) required for the recognition by the thioesterase.

Palmitoylation of several GPCRs, including β_2- and α_{2A}-adrenergic, dopamine D1, muscarinic acetylcholine m2 and 5-$HT_{4(a)}$ receptors, have been shown to be regulated by the agonist (Bouvier et al., 1995b; Hayashi and Haga, 1997; Kennedy and Limbird, 1994; Loisel et al., 1996; Ng et al., 1994; Ponimaskin et al., 2001). On the contrary to these data, the agonist stimulation of the recombinant 5-HT_{1A} receptor did not cause any changes in its palmitoylation efficiency (Fig. 4.4). Since it has been reported that the recombinant 5-HT_{1A} receptor couples effectively to the endogenous G_o-like proteins in the insect cells (Mulheron et al., 1994), we suggest that the agonist-independent palmitoylation obtained here reflects the real physiological situation. Moreover, the results obtained after the co-infection of the Sf.9 cells with the recombinant Gi-protein are further confirming the agonist-independence of the 5-HT_{1A} receptor palmitoylation also in the coupled system.

5.2. Sites of the 5-HT_{1A} Receptor Palmitoylation

From the analysis of the palmitoylated GPCRs, it is known that palmitoylation occurs on the cystein residues located in the C–terminal juxta-membrane region of the receptors (Qanbar and Bouvier, 2003). The 5-HT_{1A} receptor possesses two cystein residues within its C-terminal cytoplasmic domain, Cys417 and Cys420. These residues are conserved among the human, the rat and the murine 5-HT_{1A} receptors (Charest et al., 1993). The replacement of the both Cys417 and Cys420 by serines resulted in the non-palmitoylated 5-HT_{1A} receptor (Fig. 4.5). The replacement of the single cystein residues

Cys417 or Cys420 led to an approximately 80% reduction of the palmitoylation, when compared with the wild type 5-HT_{1A} receptor, suggesting the positive cooperativity between the palmitoylation of the Cys417 and the Cys420 residues.

In order to gain better insights into the molecular mechanisms of the 5-HT_{1A} receptor signaling and to search for the specific biological functions, we further analysed the palmitoylation-deficient mutants for their ability to activate the 5-HT_{1A} receptor specific signaling pathways.

5.3. Functional Role of the 5-HT_{1A} Receptor Palmitoylation

Multiple studies of the palmitoylated GPCRs did not reveal a common function for the receptor palmitoylation. It has been shown that palmitoylation may be involved in several aspects of the GPCR function, from ensuring the expression of functional receptors on the cell surface (Blanpain et al., 2001; Fukushima et al., 2001) to various aspects of the signal transduction (Hayashi and Haga, 1997; O'Dowd et al., 1989; Okamoto et al., 1998b) and the receptor desensitization and downregulation (Bradbury et al., 1997; Eason et al., 1994; Hukovic et al., 1998; Kraft et al., 2001). Therefore we analysed the possible functional role of the 5-HT_{1A} receptor palmitoylation for the coupling with the heterotrimeric G-proteins and for the modulation of the downstream signaling cascades, including the stimulation of the mitogen-activated protein kinase (Erk2) mediated by $G\beta\gamma$ subunit and the inhibition of the forskolin-stimulated cAMP formation mediated by the $G\alpha_i$ subunit.

Characterization of the acylation-deficient 5-HT_{1A} mutants revealed that the palmitoylation at either the Cys417 or the Cys420 residue was still sufficient to maintain the interaction of the mutant receptors with the G_i-protein in the membranes of the Sf.9 cells coexpressing the receptor and the G_i protein, although to a significantly lower extent than the WT receptor (Fig. 4.6, 4.8 and 4.9). Mutation of the both palmitoylation sites completely abolished coupling of the receptor with the G_i protein, indicating that the palmitoylation of the 5-HT_{1A} receptor is critically involved in the signal transduction. The importance of the palmitoylation for the signal transduction by the 5-HT_{1A} receptor was further confirmed by the analysis of the receptor-mediated inhibition of the forskolin-stimulated cAMP formation (Fig. 4.8) and the Erk 1/2 phosphorylation (Fig. 4.9)

The data on the important functional role played by the 5-HT_{1A} receptor palmitoylation for the signal transduction are consistent with the recent reports demonstrating the importance of the palmitoylation of the β2-adrenergic and the endothelin-B receptors for the agonist-stimulated coupling to $G\alpha_s$ and to both $G\alpha_q$ and $G\alpha_I$ proteins, respectively (Moffett et al., 1993; O'Dowd et al., 1989; Okamoto et al., 1998b). The recent data on the CCR5 and the prostacyclin receptors also demonstrated that the receptor palmitoylation is significantly involved in the G protein coupling and the effector signaling (Blanpain et al., 2001; Miggin et al., 2003). On the other hand, this is in contrast with the recent results obtained for the 5-$HT_{4(a)}$ receptor. The palmitoylation of this receptor was shown to regulate the constitutive receptor activity and was not critically involved in the coupling between the receptor and the G_s-protein after the agonist-stimulation (Ponimaskin et al., 2002a). Similar results have been also reported for the α2-adrenergic receptor which remained functionally coupled to the G_i protein after the

elimination of the palmitoylation site (Kennedy and Limbird, 1993). In the case of the μ2-muscarinic receptor, it has been shown that the C-terminal Cys457 is not required for the receptor-mediated inhibition of the AC activity (van Koppen and Nathanson, 1991), although its mutation decreases the ability of the receptor to activate the G_i protein (Hayashi and Haga, 1997). These opposing findings demonstrate that there is no common function for the acylation applicable to all GPCRs, and a thorough analysis of each individual receptor is therefore necessary.

How can the palmitoylation of the 5-HT_{1A} receptor mediate the communication between the receptor and the G_i protein? Since the subcellular distribution (data not shown), the surface expression level and the pharmacological properties of palmitoylation-deficient mutants (Fig. 4.7 and table 4.1) are quite similar to those obtained for the receptor wild type, we exclude the differences in the intracellular receptor trafficking and the agonist binding as possible reasons for the impaired G_i protein activation. Alternatively, there are two ways in which palmitoylation could affect the receptor functions: (***i***) the palmitoylation may be required to assume the proper conformation of the 5-HT_{1A} receptor needed either for the receptor/G protein recognition and the binding process and/or for the receptor-mediated G protein activation or (***ii***) the palmitoylation may be essential for the receptor localization in the specific membrane subdomains, like the lipid rafts.

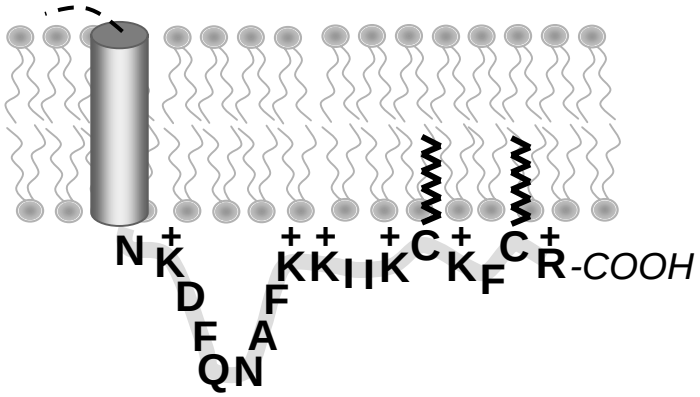
It has been proposed that the palmitoylation of the GPCRs may provide a lipophylic membrane anchor to create an additional fourth intracellular loop in the carboxyl-terminal region of the receptor (Bouvier et al., 1995a; Ross, 1995). More recently, direct evidence for this idea has been obtained for the rhodopsin (Moench et al., 1994; Palczewski et al., 2000). Since the 5-HT_{1A} receptor possesses the double acylation site within the C-

terminal domain (Fig. 4.5), the complete receptor palmitoylation may result in the formation of the additional small intracellular loop as proposed in Fig.5.1. The fact that the 5-HT_{1A} receptor remains in the continuous palmitoylation state suggests the tight and irreversible association with the plasma membrane. Such membrane association may be further stabilized by basic amino acids surrounding the palmitoylated cystein residues (Fig. 5.1). According to the two-signal model for membrane binding (Resh, 1999; Wedegaertner, 1998), the combination of the palmitate and basic motif may provide stable and essentially irreversible binding of the intracellular C-terminal domain with the plasma membrane. Functionally, the resulting conformation of the C-terminal domain may represent the structural determinant important for the communication with G_i proteins. Mutation of the single acylation site will result in the more transient interaction of the receptor C-terminus with the membrane. Although such conformation will be still sufficient for the interaction with the G_i protein in some extent, the general coupling efficiency will be reduced. Replacement of both palmitoylated cysteines may destroy the fourth loop and therefore abolish the G_i-mediated receptor activity.

An intriguing alternative mechanism could be the involvement of the palmitoylation in trafficking of the 5-HT_{1A} receptor to specific membrane subdomains. Palmitoylation has been reported to be important for the enrichment of several acylated proteins in the detergent-resistant membranes (DRM), like the caveolae and the lipid rafts (Fragoso et al., 2003; Hiol et al., 2003; Kalinina and Fricker, 2003; Li et al., 2002; Melkonian et al., 1999; Moffett et al., 2000; Niethammer et al., 2002; Patterson, 2002). Assuming that the palmitoylation of the 5-HT_{1A} receptor may represent a signal for the DRM trafficking, it could be suggested that the removal of one or both palmitate chains from the 5-HT_{1A} receptor will reduce its association with the DRMs. Indeed, our preliminary data on the

cell fractionation suggest that approximately a 30 % of the wild type 5-HT_{1A} receptor is localized in the TritonX-100 insoluble, low density membrane fractions together with the G_i protein (Fig. 4.10). Moreover, the presence of the 5-HT_{1A} receptor in the DRM fractions was reduced upon the cholesterol depletion (Fig. 4.11), confirming that the partial buoyancy of the 5-HT_{1A} receptor could be due to its localization in the cholesterol-enriched plasma membrane fractions, like the lipid rafts or the caveolae.

Figure 5.1. Proposed Structure for the C-terminal Domain of the 5-HT_{1A} Receptor. The seventh transmembrane domain as well as the amino acid sequence of C-terminal cytoplasmic tail of the 5-HT_{1A} receptor are shown schematically. The basic residues are marked by "+". A cluster of basic residues can provide electrostatic interaction with the inner leaflet of the membrane bilayer. Two palmitate moieties provide additional hydrophobic interaction with membrane, therefore resulting in more persistent association. In combination, these two signals can contribute to the formation of a stable intracellular loop.



In contrary, the nonpalmitoylated C417/420-S mutant was significantly depleted from the low density fractions, being represented there only by approximately 4% of the total receptor amount (Fig. 4.10). Since the detergent-resistant membrane subdomains have been proposed to maintain the different components of a particular signaling system

together, therefore representing the “hot spots” for signaling (Huang et al., 1997), the non-DRM localization of the 5-HT_{1A} receptor may result in the uncoupling from the G_i/adenylate cyclase signaling pathway. Indeed, partial localization in the DRM was shown to be critical for the signal transduction by several G protein coupled receptors. For example, it has been shown that the small fraction (10%) of the endothelin B (ET_B) receptor is more resistant to the ligand-promoted internalization the non-DRM localized ET_B receptors, thus allowing it to couple to specific signaling pathways (Chini and Parenti 2004). Additional studies, involving novel state-of-art methods for the investigation of the plasma membrane subdomains like the co-patching of the membrane components by external crosslinking agents (Harder et al., 1998) and electron microscopy are necessary to establish whether the stable palmitoylation of the 5-HT_{1A} receptor is involved in the partial DRM trafficking and confers the colocalization of the receptor with its signaling partners.

6. SUMMARY

In the present study, we demonstrated that the mouse 5-hydroxytryptamine (1A) (5-HT_{1A}) receptor is modified by the palmitic acid, that is covalently attached to the protein through the thioester-type bond. The palmitoylation efficiency was not modulated by the receptor stimulation with the agonist. Block of the protein synthesis by cycloheximide resulted in the drastic decrease of the receptor acylation, suggesting that the palmitoylation occurs early after the synthesis of the 5-HT_{1A} receptor. Furthermore, the pulse-chase experiments demonstrate that the fatty acids are stably attached to the receptor.

Two conserved cystein residues C417 and C420 located in the cytoplasmic C-terminal domain were identified as the palmitoylation sites by the site-directed mutagenesis. To address the functional role of the 5-HT_{1A} receptor palmitoylation, we analysed the ability of the palmitoylation-deficient mutants to interact with the heterotrimeric G_i protein and to modulate the downstream effectors. Replacement of the individual cystein residues (C417 or C420) resulted in the significantly reduced coupling of the receptor with the G_i protein and in the impaired inhibition of the adenylate cyclase activity. When both palmitoylated cysteines were replaced, the communication of the receptors with the G_i proteins was completely abolished. Moreover, the non-palmitoylated mutants were no longer able to inhibit the forskolin-stimulated cAMP formation, indicating that the palmitoylation of the 5-HT_{1A} receptor is critical for the interaction with the heterotrimeric G_i proteins. The receptor-dependent activation of the extracellular signal-regulated kinase was also affected by the acylation-deficient mutants, suggesting the importance of the receptor palmitoylation for the signaling through the Gβγ-mediated

pathway, in addition to the $G\alpha_i$-mediated signaling. In addition, we demonstrated that the palmitoylation may be involved in the partial targeting of the 5-HT_{1A} receptor to the detergent-resistant membrane subdomains.

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Publications having a relation to this thesis are marked by *.

Papers:

* **Papoucheva E,** Dumuis A, Sebben M, Richter DW, Ponimaskin EG. The 5-hydroxytryptamine(1A) receptor is stably palmitoylated, and acylation is critical for communication of receptor with Gi-protein. Journal of Biological Chemistry. 2003 Jan. 30, V. 279, pp. 3280-3291.

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